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The Endogenous Galactose-Specific Lectin in Early Development of *Xenopus Laevis*.

By

HARRIET L. HARRIS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

FALL, 1986

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DEDICATION

This thesis is dedicated to Lorena and Timothy.

ABSTRACT

This study describes an endogenous lectin present in early stages of Xenopus laevis embryos. Extracts of embryos at cleavage, blastula, gastrula and neurula stages contained lectin activity which agglutinated trypsinized rabbit erythrocytes. This activity was found to be significantly higher in gastrula embryos compared to the levels found in embryos at cleavage, blastula and neurula stages. The highest activity, therefore, correlated with the stage of active morphogenetic movements. Lectin-mediated agglutination required the presence of calcium and was inhibited by α- and β-galactosides. The best soluble inhibitors of haemagglutination were lactose and thiodigalactoside. When saccharides bound to an insoluble matrix were tested, the disaccharide DGal(α1-3)DGalβ1-R was the most potent inhibitor.

Lectin activity was purified by batch adsorption to αGal-Immunoadsorbent or affinity chromatography on a column containing Sepharose 4B linked to para-aminophenyl-β-D-lactoside. Purified lectin was examined by SDS-polyacrylamide gel electrophoresis. Samples which had not been treated with chloroform-methanol contained associated lipid-like material. This material appeared as a number of bands on SDS-PAGE, the most prominent of which

was a diffuse band at 37,000 to 45,000 molecular weight. A single band with a molecular weight of 65,500 ± 2,780 was observed when chloroform-methanol treated lectin samples were used for SDS-PAGE. Purified lectin examined by gel filtration chromatography in the presence of lactose was present as an aggregate. The size of the aggregate was 495,000 before treatment with chloroform-methanol and 371,000 after treatment, indicating that the chloroform-methanol treatment extracted some associated material from the lectin aggregate.

Purified, chloroform-methanol treated lectin was used to raise antiserum in rabbits. The antiserum, exposed to crude or purified lectin showed a single precipitin line in agar diffusion tests. It also inhibited lectin-mediated haemagglutination. The lectin protein was denatured by the sample buffer used for SDS-PAGE and did not stain with antiserum in immunoblotting experiments. However, when dotted directly onto nitrocellulose paper, a lectin sample which appeared at 65,000 molecular weight on SDS-PAGE, stained with the antiserum.

The antiserum was used to determine the location of the lectin protein in cleavage and blastula embryos. Sectioned embryos were studied by immunofluorescence or immunohistochemistry using antibody bound to glucose oxidase-antiglucose oxidase complex. The lectin appeared

in the vitelline membrane, underneath the vitelline membrane on the blastomere surface, in the blastocoele cavity, within cleavage furrows and between membranes of adjacent cells. In some sections, stain was observed in intracellular deposits which may have been associated with yolk granules or lectin in the process of being externalized. At this time, the localization reflects the distribution of the 65,000 molecular weight lectin and the 37,000 to 45,000 molecular weight associated material.

The distribution of lectin, together with the fact that lectin activity is elevated at the gastrula stage, suggests a role for this molecule in cellular interactions during morphogenetic movements in Xenopus embryos. Possibly, the externalized molecule may be a component of the extracellular matrix or it may modify adhesive interactions between blastomeres. Alternatively, an intracellular role in organization or utilization of yolk cannot at this time be excluded.

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INTRODUCTION:

1. THE AMPHIBIAN EMBRYO

1.1 The Organization of the Egg:

The unfertilized egg of Xenopus laevis is a spherical cell approximately 1.2-1.4 mm in diameter (Gerhart, 1980). It already has an evident polarity shown by the darkly pigmented upper hemisphere (the animal pole) and the light lower hemisphere (the vegetal pole). This polarity is due to the unequal distribution of cytoplasmic components. At least four internal regions of the egg can be distinguished on the basis of cytoplasmic organization. The cortex is the area underlying the plasma membrane which is devoid of yolk platelets but contains numerous pigment granules, cortical granules and actin-containing microfilaments. The deeper cytoplasm of the animal pole contains loosely-packed, small yolk platelets (2-4 μm) and pigment granules. The deep cytoplasm of the vegetal pole contains large, more concentrated yolk platelets (10-14 μm) but pigment granules are less numerous in this region. Between the animal and vegetal hemispheres a transitional area exists known as the marginal zone. It is characterized by the presence of intermediate-size yolk platelets and an intermediate number of pigment granules.

The yolk platelets are ovoid, membrane-bound structures thought to originate from the Golgi complex and the endoplasmic reticulum (Kress and Spornitz, 1972). They consist of a central crystalline matrix surrounded by more amorphous material (Wischnitzer, 1966). In the early stages of oogenesis, yolk proteins and lipids are synthesized in the oocyte. Later on the precursor yolk protein vitellogenin is synthesized in the liver and transported to the oocyte (Dumont, 1978). The breakdown products of vitellogenin include a lipophosphoprotein, lipovitellin and a phosphoprotein, phosvitin (Wallace and Bergink, 1974). These proteins accumulate in small yolk platelets which later fuse together to form larger platelets (Karasaki, 1963).

Cortical granules are produced in the endoplasm of the egg and migrate to the cortex as the egg matures. Precursors of cortical granules are membrane-bound organelles derived from the Golgi complex (Wischnitzer, 1966). The mature cortical granule contains 20% by weight neutral and sulphated glycosaminoglycans and 80% proteins (Wolf et al, 1974). The contents of the cortical granules are extruded by exocytosis from the egg membrane at fertilization.

The enlarged nucleus of the oocyte is known as the germinal vesicle. The initial stages of meiosis occur

early in oogenesis but meiosis is suspended at diakinesis of prophase I. During this phase, most of the growth and differentiation of the oocyte takes place. The chromosomes during this time are in the form of lampbrush chromosomes and considerable transcription occurs in this state. Large amounts of polyadenylated messenger RNA is stored in the cytoplasm in the form of ribonucleic acid-protein particles (RNP). These components are thought to be an information store which remain inactive in the egg until fertilization.

Maturation of the oocyte occurs in response to steroid hormones. This process has been extensively studied by Masui and coworkers and is reviewed by Masui and Clark (1979). During this stage, the germinal vesicle breaks down and the chromosomes condense and proceed to metaphase of the second meiotic division. Maturation also induces changes in ion permeability of the plasma membrane and the movement of the cortical granules to a position just underneath the plasma membrane.

The mature oocyte is released from the ovary and passes down the oviduct. It is surrounded by the vitelline envelope which is composed mainly of glycoproteins and is laid down initially as a product of the growing oocyte and the follicle cells (Dumont and Brummett, 1985). As the oocyte passes down the oviduct a 57,000 molecular

weight component is added to the vitelline envelope with the result that it changes from a fibrillar, porous membrane to a more dense structure without pores (Schmell, 1983). The added component appears to increase fertilizability of the egg. Three layers of jelly are also laid down around the egg in the oviduct. The jelly is largely composed of sulphated glycoproteins, with galactosamine, glucosamine, galactose and fucose being the major carbohydrate components (Yurewicz et al, 1975).

At fertilization, the cortical granule contents are exocytosed. At least some of the cortical granule exudate passes through the vitelline envelope resulting in a change in structure of this membrane. Wyrick et al (1974) have described a lectin in this exudate which, in the presence of Ca^{++}, combines with a sulphated, galactose-containing glycoconjugate present in the innermost jelly layer. This reaction forms an F layer which becomes the outermost component of the envelope, now known as the fertilization membrane (Schmell et al, 1983). Osmotic changes brought about by changes in the permeability of the envelope raises the fertilization membrane away from the surface of the egg, forming the perivitelline space. The above alterations are thought to provide a block to polyspermy.

1.2 Early Amphibian Development:

The unfertilized Xenopus embryo is radially symmetrical about its animal-vegetal axis. Fertilization occurs in the animal hemisphere and the point of sperm entry is marked by an accumulation of pigment. Opposite to the side of the sperm entry point a differential redistribution of pigment leads to the formation of the grey crescent. According to Gerhart (1980) the formation of the grey crescent is important only insofar as the vegetal cytoplasm is displaced. The result of this displacement is a yolk-free cytoplasm at the dorsal region. This reorganization of cytoplasmic materials induced by fertilization may determine the eventual dorsal-ventral organization of the embryo. The zygote after fertilization is bilaterally symmetrical and the first cleavage plane passes through the sperm entry point and bisects the grey crecent. The grey crescent side of the embryo becomes the dorsal side; the ventral side forms opposite the grey crescent.

The blastula stage is a period of rapid cleavage during which the mass of the embryo does not change. Mid-blastula, when the embryo has approximately 1024 cells, is marked by a transition involving several simultaneous changes. These changes include the activation of transcription, a reduction in the rate of cleavage, a loss

of synchrony of cell division and the beginning of cell motility (Newport and Kirschner, 1982). At the end of the blastula stage, the embryo consists of approximately 15,000 cells (Gerhart, 1980). The animal hemisphere is composed of several layers of small cells forming the roof of a fluid-filled cavity known as the blastocoele. The floor of the blastocoele is formed of several layers of large, yolk-laden cells (see Figure 1, stage 8). Vogt (1929) used vital dye staining to trace the ultimate fates of the cells of urodele blastulas. He was able to identify three regions: Presumptive ectoderm (including epidermis and neural plate) formed from darkly pigmented, small animal hemisphere cells, presumptive notochord and mesoderm formed from marginal zone cells and presumptive endoderm formed from the large, yolky, vegetal hemisphere cells. Xenopus blastulae may differ somewhat from the above organization. Recent studies conducted by Keller (1975, 1976, 1978 and 1984) indicate that the presumptive mesoderm cells in Xenopus are located in the deeper layers rather than on the surface of the blastula.

The first indication of gastrulation in amphibians is the appearance of the blastopore at the lower edge of the grey crescent. The blastopore is an indentation formed by the inward sinking of endoderm cells. The first cells to sink into the blastopore are known as "bottle cells"

because of their distinct shape. As gastrulation continues, cells converge on the blastopore and turn inward (involution). The layer of presumptive ectoderm overlying the blastocoele thins and increases in surface area (epiboly), spreading vegetally to replace involuting endoderm cells (Keller, 1975). At the same time, presumptive mesoderm and notochord cells of the dorsal marginal zone undergo involution beneath the surface ectoderm cells. The blastopore edges extend in an arc, eventually forming a complete circle ventrally. The endoderm cells encircled by the blastopore form the yolk plug. These cells are withdrawn into the interior later in gastrulation when the blastopore closes.

The role of bottle cells in gastrulation has been the subject of some debate lately. The classical view expressed by Holtfreter (1944) is that the bottle cells pull adjacent cells into the interior. However, Cooke (1975) and Keller (1981) demonstrated that removal of the bottle cells before or during gastrulation does not prevent the subsequent cellular movements of gastrulation. If, on the other hand, deep cells of the dorsal marginal zone are replaced by cells from the animal pole or are rotated 90° with respect to their original orientation, involution and epiboly do not occur. Keller believes that the force-

generating process responsible for the movements of gastrulation originates from the mesodermal cells of the dorsal marginal zone. "Medio-lateral interdigitation" of these cells is the force which drives epiboly and involution (Keller, 1985).

At the present time, not much is known about the cellular and molecular events responsible for the movements of gastrulation. Amphibian embryos at the gastrula stage incorporate radioactively-labelled glucosamine and galactose into glycoconjugates which become predominantly localized in the extracellular matrix material (Johnson, 1984). Also, hybrid Rana embryos defective in the synthesis or secretion of this material are unable to complete gastrulation (Johnson, 1977). In amphibians, the movement of mesodermal cells along the blastocoele roof is an essential part of gastrulation. Johnson (1984) found that radioactively-labelled galactose is accumulated in Xenopus laevis embryos on the roof of the blastocoele cavity, within the blastocoele and around migrating mesodermal cells. The appearance of this material viewed in the scanning electron microscope changes from a granular form in the blastula stage to a more fibrillar form in gastrula embryos (Nakatsuji and Johnson, 1983; Johnson, 1984). Further studies by Nakatsuji et al (1982) and Nakatsuji (1984) examined the arrangement of these fibers on the

inner surface of the ectodermal layer overlying the blastocoele cavity. They concluded that the fibers were aligned along an axis running from the blastopore to the animal pole in these embryos. These authors believe that the direction of the migrating mesodermal cells along the roof of the blastocoele is determined by contact guidance provided by these fibers. Although the biochemical nature of these fibers is not known, it is possible that the galactose-containing glycoconjugates referred to above may be important components of these structures.

A recent study by Boucaut et al (1984, 1985) indicates that fibronectin is an important functional component of the extracellular material located on the inner roof of the blastocoele. In both Pleurodeles (Boucaut et al, 1985) and Xenopus (Boucaut et al, 1984), monoclonal antibodies raised against fibronectin inhibited gastrulation, indicating that this glycoprotein is necessary for cell attachment and migration along the blastocoele roof. In addition, these authors have identified a decapeptide from the cell-binding domain of fibronectin. When injected into Xenopus blastulae at a concentration of 10 µg/µl, this decapeptide inhibited gastrulation.

At the end of gastrulation, the yolk plug disappears and the nervous system begins to develop from the dorsal ectoderm. The dorsal lip of the blastopore, named the

"organizer" by Spemann and Mangold (1924) seems to have a special influence on this process. At the end of gastrulation, this tissue establishes the axial organization of the embryo by inducing neural structures to form from overlying ectoderm cells. The result is the formation of the neural tube which is the rudiment of the central nervous system. During the neurula stage, the embryo also undergoes internal reorganization and anterior-posterior elongation. The archenteron, a large internal cavity lined by endoderm, becomes the primitive gut. The notochord becomes the cylindrical supporting rod and separates blocks of mesoderm on either side of it. Dorsal mesoderm becomes somite tissue and lateral mesoderm splits and forms the coelom. Cells, originating from the neural crest begin to migrate laterally and ventrally. These cells give rise to a number of cell types in the later embryo, including pigment cells, sensory and autonomic ganglia, cartilage, connective tissue and Schwann cells.

Although cell displacement and shape changes do occur during the neurula stage, the major morphogenetic movements responsible for the formation of the germ layers have been completed by the end of gastrulation. The tissues brought into juxtaposition during gastrulation now begin to differentiate, in some cases in response to

induction from other tissues. The role of glycoconjugates and the extracellular matrix in these processes is now being investigated (Slack, 1985; Goulandris et al, 1985).

2. THE CELL SURFACE AND CELL ADHESION

Cell adhesion is the process whereby cells attach to one another. It is important in embryonic development because embryonic cells during morphogenesis are continually modifying their adhesive properties. Original observations by Moscona and Moscona (1952) and Townes and Holtfreter (1955) established that embryonic tissues can be dissociated into suspensions of single cells and the aggregation of those cells can be studied *in vitro*. Since then, the promotion or inhibition of aggregation in cell culture under a variety of conditions has been investigated (reviewed by Trinkaus, 1984). In these experiments, cell adhesion is identified as the formation of stable bonds between cells or between cells and the substratum.

An important aspect of adhesion in studies involving embryonic tissue is the idea of specificity. Specificity is the preferential adhesion of a cell to one cell type over another. In many experiments with embryonic cells from different tissues, the cells initially stick together non-specifically but eventually they sort out into islands of similar cells within the aggregate (Moscona, 1962;

Steinberg, 1964; reviewed in Trinkaus, 1984). These results suggest that cell recognition operates within cell aggregates resulting in tissue-specific or cell-specific sorting out (Moscona and Hausman, 1977). It is becoming clear that cell recognition involves cell surface components although the complexity and diversity of the process indicates that adhesion and sorting out are unlikely to be explained by a single molecular mechanism.

The importance of cell surface carbohydrates in adhesion was recognized by Roseman (1974) who hypothesized that transient attachments between cells were the result of the action of a glycosyltransferase on its substrate (reviewed by Shur, 1982). The result of this reaction would be the alteration of a terminal glycosyl residue and the ultimate dissociation of the interacting cells. Although there is no conclusive proof that glycosyltransferases directly mediate cell adhesion, it is now recognized that cell-to-cell and cell-to-substratum attachments are certainly influenced by the complex carbohydrates located on the cell surface; that is, glycoproteins, glycolipids and glycosaminoglycans.

It is not possible in this Introduction to provide an exhaustive review of all the cell surface molecules currently being investigated. Some of the molecules which have been implicated in cell adhesion and which pertain to

the work described in this thesis will be briefly reviewed here.

2.1 The Extracellular Matrix:

The extracellular spaces of most adult vertebrate tissues contain an extracellular matrix (ECM) consisting of protein and carbohydrate molecules assembled into an organized meshwork. Highly specialized structures such as cartilage, tendons and basement membranes are formed from extracellular matrix materials. In addition, nearly all of the cells of a multicellular organism are coated with a thin layer of material containing carbohydrates. The extracellular material becomes organized in the vertebrate embryo at about the time of gastrulation (Hay, 1981). In embryos, loosely organized mesenchyme contains abundant ECM. In avian embryos, migrating mesenchymal cells move under the ectoderm along a basement membrane during primitive streak formation. Similarly, in amphibian embryos, as mentioned previously, ECM is detected on the roof of the blastocoele during mesodermal cell migration (Johnson, 1984).

Basal laminae (otherwise known as basement membranes) are mats of extracellular matrix material which generally underlie endothelium, smooth muscle cells and epithelium (Farquhar, 1981). They occur in the form of a meshwork of

fine fibrils and serve as an anchor for the overlying cells. Collagen and other non-collagenous glycoproteins such as laminin and fibronectin are major components of the basement membranes. Proteoglycans also occur in these structures (Hascall and Hascall, 1981) as do glycosaminoglycans (Toole, 1981).

Interest in the molecules comprising the ECM has been stimulated by the observation that they may serve as a substratum for cell migrations *in vivo*. Materials isolated from the ECM in some cases specifically promote the adhesion of cells or their attachment to the substratum. Some of these molecules are described briefly below.

2.1.1 Collagen.

Collagen is the major structural protein of the ECM. It provides tensile strength to the connective tissue and is a major component of cartilage and bone (Hewitt and Martin, 1984). Collagen molecules are glycoproteins, having a variable number of attached sugar residues (Mayne, 1984). Collagen molecules exist in their native state as a triple helix containing three polypeptide chains. The conformation is stabilized by interchain hydrogen bonds.

Collagen occurs in tissue-specific patterns and different forms are produced at different developmental times (Miller, 1977). At least five kinds of collagen have been described. These forms differ in their biochemistry and distribution. Basal laminae possess both Type IV and Type V collagens (Linsenmayer, 1981). These collagens are found in high levels in lens capsule, kidney glomerulus and human placenta.

Collagen polymerized in the form of three-dimensional gels serves as an adhesive substratum for cells and promotes cell migration (Yamada, 1983a). The effect of collagen on cell adhesion depends on the interaction between this molecule and other ECM components. For example, collagen was found to inhibit the spreading of BHK cells on a fibronectin-coated substratum (Nagata et al, 1985). Collagen also binds proteoglycans and laminin (Type IV collagen) in addition to fibronectin (Hewitt and Martin, 1984).

Collagen matrices promote the growth and differentiation of cells in culture (Hay, 1981). For example, <u>in vitro</u> differentiation of myoblasts to myotubes has been shown to depend upon the presence of a collagenous substratum (Ketley et al, 1976).

The effects of collagen on cell adhesion has been recently reviewed (Hewitt and Martin, 1984).

2.1.2 Fibronectin.

Fibronectin is a glycoprotein found on the cell surface of many cells, in the extracellular matrix and in the plasma. It is a dimer of two similar subunits, each having a molecular weight of approximately 230,000, with 5% carbohydrate. The structure of the fibronectin molecule has been recently reviewed by Hynes (1985). It aggregates to form fibrillar networks around those cells which secrete it. Both the dimer and the aggregate are held together by disulphide bridges. Fibroblasts, myoblasts, endothelial cells and amniotic cells all produce fibronectin in culture. The secreted molecule becomes localized between adjacent cells and between cells and their substratum. Some cells, including transformed cells, moving cells, cells in mitosis and neurons appear to have very little fibronectin. Fibrils of fibronectin are often left behind when cells are detached from the substratum. The fibronectin molecule has binding sites for the cell surface and also for fibrin, collagen and sulphated

proteoglycans (Furcht, 1983; Yamada, 1983a). The evidence that fibronectin is a cell adhesion-promoting molecule comes from the laboratories of Hynes (Hynes, 1981) and Yamada who have shown that isolated, electrophoretically pure fibronectin promotes the non-specific aggregation and attachment of several types of cells including transformed fibroblasts. Fibronectin appears to act as a multivalent ligand. Also, when healthy, attached cells in culture are treated with anti-fibronectin antibodies, there is a rapid withdrawal of cell extensions, a rounding of cells and a detachment from the substratum (Yamada, 1983 a and b).

2.1.3 Laminin.

Laminin is a large glycoprotein (molecular weight 1 x 10^6) which is a major component of the basement membrane (Hewitt and Martin, 1983). It is composed of one large polypeptide chain (molecular weight 440,000) and three smaller chains (molecular weight 220,000) held together by disulphide bonds. Laminin binds to Type IV collagen and also has binding domains for certain glycosaminoglycans, including heparin and heparan sulphate (Kleinman et al, 1985). By binding to these basement membrane components,

laminin may help to organize the matrix of the basement membrane. Both the large and the small polypeptide chains have been described in mammalian embryos as early as the 16-cell stage (Leivo et al, 1980). Like fibronectin, laminin appears to be an attachment molecule. Localization studies have determined that laminin is present in the basement membrane adjacent to the basal surface of epithelial cells (Hewitt and Martin, 1984). Receptors for laminin are present on epithelial, muscle and tumor cell surfaces (Kleinman et al, 1985). The role of laminin seems to be to attach epithelial cells to the basement membrane.

2.1.4 Glycosaminoglycans.

Glycosaminoglycans (GAG's) are carbohydrate polymers of high molecular weight composed of repeating amino sugars and uronic acids. Examples of GAG's are hyaluronic acid, chondroitin, chondroitin sulphate, heparin, heparan sulphate and keratan sulphate. These large molecules are further complexed to proteins resulting in proteoglycans. Their complexity is almost limitless. GAG's occur at the surfaces of cells and have been postulated to be

involved in cell contact phenomena (Hook, 1984). GAG's interact with other molecules of the ECM and possibly influence the binding of cells to fibronectin or collagen. Heparan sulphate is an integral membrane component in hepatocytes, fibroblasts and endothelial cells (Bernfeld et al, 1984). A possible function of this GAG is to link intracellular and extracellular fibers by interacting with both actin filaments in the cytoplasm and externally with ECM components. In support of this idea, heparan sulphate proteoglycans are located in focal adhesion sites of rat fibroblasts in culture. These sites, described by Singer (1979) are special regions on the underside of cells which form tight attachments to the substratum. As well as glycosaminoglycans, this region also contains fibronectin.

2.2 Aggregation Promoting Factors:

Moscona and his coworkers began the isolation of soluble factors which promote the aggregation of cells in culture in a tissue-specific manner (Lilien and Moscona, 1967). Since then, such factors have been described in many systems, including embryonic nervous system (Hausman and Moscona, 1976), teratocarcinomas (Meyer and Oppenheimer, 1975) and embryonic liver (Sankaran, 1977).

In spite of extensive work, it is not yet clear whether these molecules are true components of a cell adhesion mechanism or only indirectly influence cell adhesion.

2.3 Cell Surface Antigens:

One recent approach to the problem of identifying molecules involved in cell attachment and aggregation is to prepare antibodies against cell surface molecules. If the molecules are involved in cell attachments, then antibodies raised against them, in the form of monovalent Fab fragments, should inhibit cell aggregation. This aproach was first used by Gerish to investigate slime mold aggregation (reviewed in Section 3.5.3), (Huesgen and Gerish, 1975). Edelman and coworkers have used this method to describe two cell adhesion molecules (CAM). One of these is a large surface sialoglycoprotein isolated from neural retinal cells and called N-CAM (Brackenbury et al, 1977; Thiery et al, 1977). This molecule is an integral membrane protein with a molecular weight between 120,000 and 140,000. In addition to neural retinal cells, it is found on the surface of brain and muscle cells of the chick and rat (Edelman, 1983). Monovalent antibodies prepared against this molecule inhibited the side-to-side attachment of neurites, which is a necessary step in the formation of nerve fascicles (Rutishauser et al, 1978).

Also, neural retinas from 6-day chick embryos placed in culture in the presence of anti-N-CAM were disrupted in their tissue organization and lacked sharp boundaries. Compared to control cultures, there were few areas of cell-to-cell contact in the treated retinas and large intercellular spaces (Buskirk et al, 1980).

A second cell adhesion molecule L-CAM, has been found in chick and rat liver tissue (Bertolotti et al, 1980). The hypothesis has been put forward that both L-CAM and N-CAM are primary binding molecules which attach cells together (Edelman, 1985). However, since N-CAM itself does not appear to block cell adhesion of neural retinal cells, the situation may be more complex than this hypothesis indicates. The precise role of the CAM's remains to be determined.

3. LECTINS

3.1 Definition of Lectins:

The name lectin, from the Latin word "legere" meaning to choose, defines a class of proteins or glycoproteins which can selectively agglutinate vertebrate erythrocytes because of their binding properties for specific cell surface carbohydrates. Lectins are divalent or polyvalent proteins and binding of these molecules to erythrocyte

surfaces leads to agglutination of the erythrocytes. Thus, lectins are also known as agglutinins or haemagglutinins. The most widely used method for identifying lectin activity is the haemagglutination test. The specificity of a lectin is normally defined with respect to the monosaccharide which most effectively inhibits its agglutinating activity.

Lectins were initially isolated from seed plants. In 1888, Stillmark discovered that extracts of the castor bean Ricinus communis could agglutinate animal erythrocytes (reviewed in Goldstein and Hayes, 1978). Since then, lectins have been described in a wide variety of plant species (Jermyn and Yeow, 1975; Lis and Sharon, 1981). Interest in the properties of lectins was greatly enhanced by three separate discoveries. Firstly, Landsteiner discovered that erythrocyte agglutination by some plant lectins was blood-group specific. This specificity is in most cases related to the major carbohydrate determinate on the surface of the erythrocyte (Boyd and Shapleigh, 1954). The second discovery, by Nowell (1960), was that some plant lectins have a mitogenic effect on various lymphocyte cells. In particular the lectin from the jack bean, concanavalin A (Con A) and phytohaemagglutinin (PHA) from Phaseolus vulgaris stimulated mitosis

in thymus-derived lymphocytes (T cells) but not bone marrow-derived lymphocytes (B cells) (Blomgren and Svedmyr, 1971). The third important discovery, made by Aub and coworkers in 1963, was that extracts from Triticum vulgaris agglutinated transformed cell lines but did not agglutinate the corresponding untransformed cells. The causative agent was found to be a lectin, wheat germ agglutinin (WGA) (Burger and Goldberg, 1967). These discoveries led to the widespread use of plant lectins as probes for investigating normal and tumor cell surfaces, cell surface glycoproteins and the structure of blood group substances (Nicholson, 1974; Sharon and Lis, 1972).

The function of these lectins in the plant tissues from which they are isolated remains obscure (for review, see Goldstein and Hayes, 1978; Toms and Western, 1971). Recent work by Dazzo and coworkers (Dazzo et al, 1982) suggests that a lectin functions in the attachment of the symbiont Rhizobium to root hairs of the clover, Trifolium repens.

The studies with plant lectins led to an interest in endogenous lectins in other biological groups. Since then, lectins have been described in many bacterial, animal and fungal species.

3.2 Lectin Binding Properties:

The saccharide binding specificity of a lectin is normally determined by testing various α- and β-glycosides, free sugars, di- and higher oligosaccharides and cell surface glycoproteins for the ability to inhibit lectin-mediated agglutination. The binding between lectins and their sugars is generally thought to involve hydrogen bonds and non-polar interactions.

According to Goldstein and Hayes (1978) the binding site of most well-studied plant lectins accommodates a single glycosyl residue. Lectins interact primarily with the nonreducing terminal groups of polysaccharides and glycoproteins but, in many cases, the nature of the glycosidic linkage of the terminal sugar and its anomeric configuration are also important. Alkyl α- and β-glycosides are frequently used as inhibitors to provide information about the anomeric specificity of a lectin. Some lectins show a preference for one anomeric form over the other. Con A, for example shows a specificity for α-D-mannose or α-D-glucose. Other lectins do not exhibit such a specificity. For example, soybean agglutinin (SBA) will bind equally well to α-D-galactosamine or β-D-galactosamine.

It also appears from saccharide inhibition studies that the carbon-2 position of the sugar is not critical in

determining the specificity of many plant lectins (Lis and Sharon, 1981). Thus, Con A shows a specificity for α-D-mannose and also binds well to α-D-glucose and N-acetylglucosamine. Variations at the carbon-3 and carbon-4 positions, however, have a more crucial effect on lectin binding. Lectins which bind galactose, for example, do not normally interact with mannose or glucose and vice-versa.

Plant lectins vary also in their requirements for bound metal ions. Some lectins, such as Con A and PHA, require both Mn^{++} and Ca^{++} for full activity. Others require only Mn^{++} (lentil lectin) or Ca^{++} (pea lectin) for activity, while other lectins have no requirement for metal ions, such as WGA (Lis and Sharon, 1981). Where metal ions are necessary for lectin activity, they probably cause a conformational change which creates or stabilizes the saccharide binding site (Doyle et al, 1975).

The binding of lectins to glycoproteins and cell surface glycoconjugates often occurs with higher affinity than the binding of the same lectin to simple sugars. There are several possible reasons for this observation. Lectins are capable of forming multivalent bonds to their ligands and glycoproteins or glycoconjugates may provide multiple binding sites. Thus, lectins may bind well to

clusters of saccharides although they might not interact with high affinity to the same carbohydrate in a monosaccharide form. Lee and coworkers (1983) have observed this effect with the mammalian hepatocyte lectin. This lectin binds strongly to ligands bearing polyvalent clustered galactose residues but the binding to monovalent galactosides is relatively weak. In addition to this cluster effect, it is likely that some lectins can bind to internal sugar groups as well as terminal ones. Con A is known to bind internal, mannopyranosyl groups and WGA also interacts with internal residues (Lis and Sharon, 1981). And finally, non-specific interactions and steric factors probably play an important role in the attachment of lectins to macromolecules (Goldstein and Hayes, 1978). Since cell surface receptors for lectins are most likely complex oligosaccharides, all of the above factors are expected to influence the agglutination reaction and also the interaction of lectins with their endogenous receptors.

3.3 The Occurrence of Lectins in Invertebrates:

Lectins have been discovered in all of the major classes of invertebrates (Yeaton, 1981). They are most often found in high concentrations in haemolymph but can also be isolated from whole body extracts or tissue

extracts. A single invertebrate species frequently contains more than one lectin which may differ in molecular weight, carbohydrate specificity, isoelectric points or mitogenic properties. Lectins isolated from invertebrates show considerable diversity in their sugar specificity, including some which are specific for glucose, galactose, fucose, N-acetyl neuraminic acid, N-acetylglucosamine and N-acetylgalactosamine. In other words, all of the monosaccharides present on the surface of mammalian erythrocytes are recognized by invertebrate lectins with the exception of mannose. Indeed, since vertebrate erythrocytes are used to assay for the presence of invertebrate lectins, many lectins which bind to sugars not present on these erythrocytes may go undetected. Some invertebrate lectins appear to be developmentally regulated since the level of activity changes at different stages of the life cycle. For example, in the silk moth, Hyalophora cecropia a major lectin present in the hemolymph peaks in activity from the fourth instar to the adult stage but it is not detectable in the eggs (Yeaton, 1981). In the opinion of Yeaton, probably all invertebrates possess lectins at some stage of their life cycle.

The function of invertebrate lectins is uncertain. One possibility advanced by several authors is that these lectins may act as a primitive immune system (Acton,

1974), perhaps as antibody-like molecules which can agglutinate bacteria, viruses or parasites by binding to the surface carbohydrates of these organisms (Marchalonis, 1978). Other possible functions suggested for invertebrate lectins are; as opsonins which enhance phagocytosis (Prowse and Tait, 1969); as a mechanism for maintaining sterility in the perivitelline space surrounding the egg (Pemberton, 1974); to mediate symbiosis (Ratanarat-Brockelman, 1977); to function in the deposition of chitin (Cohen, 1970); to regulate glycoprotein-ordered multi-enzyme complexes (Sharon and Lis, 1972) and to mediate cellular adhesion (Gold et al, 1974). These various functions have not as yet been clearly demonstrated for any invertebrate lectin, but it is possible that these molecules may play diverse roles in different species and at different stages of the life cycle.

3.4 The Occurrence of Lectins in Vertebrates:

Lectins also occur widely in vertebrate species. The situation is probably similar to the one in invertebrates in that probably all species possess lectins in some tissues at some developmental stages. In vertebrate studies, however, the assay used for testing the presence and activity of lectins involves vertebrate erythrocytes. Thus the results are more likely to be relevant to the

in vivo activity of the lectin than is the case for invertebrate lectins.

Vertebrate lectins are found as both membrane-bound and soluble proteins, and according to Barondes (1984) this difference probably reflects a fundamental difference in their function. Membrane bound lectins appear to function in the transport of glycoconjugates and their localization at particular membrane sites (Ashwell and Harford, 1982). Soluble lectins on the other hand, may play a wider role in modification of the extracellular environment thereby influencing cell-cell interactions during specific development stages. In support of this idea, many soluble lectins are developmentally regulated and their activity is maximal at times of tissue organization. As well, soluble lectins which are initially located internally in cells are later secreted outside where they become associated with external glycoconjugates (Barondes, 1984).

Some vertebrate lectins which have been studied most thoroughly and are thought to play a role in determining cell behaviour during development are reviewed below.

3.5 Lectins in Cell Recognition and Development:

3.5.1 The Mammalian Hepatic Lectin.

This protein, first described by Stockert and coworkers (1974), was the first lectin to be isolated from a mammalian species and is still the only animal lectin with a known function. The hepatic binding protein is a membrane-bound glycoprotein and can be solubilized from the membranes of rabbit hepatocytes in the presence of detergent. The oligomeric protein in aqueous solution has a molecular weight of 500,000 (Ashwell and Morell, 1977) and is composed of two subunits of 48,000 and 40,000 molecular weight. Bound carbohydrate, including sialic acid, galactose, mannose and glucosamine residues, make up 10% of the dry weight of the protein (Kawasaki and Ashwell, 1976). This lectin is specific for β-galactose and it agglutinates human and rabbit erythrocytes and neuraminidase-treated erythrocytes from guinea pig, rat and mouse (Stockert et al, 1974). Intact sialic acid residues and the presence of Ca^{++} ions are important for the activity of the lectin (Hudgin et al, 1974).

This lectin functions in the specific recognition and clearance of circulating aged glycoproteins which have lost their terminal sialic acid residues.

According to the hypothesis of Ashwell and his coworkers (Ashwell and Morell, 1974), serum glycoproteins must have an intact terminal sialic acid residue to remain in circulation. Upon the loss of this sialic acid, a β-galactose group is exposed. The asialoglycoprotein is recognized, bound and removed from the circulation by the hepatic lectin. In support of this hypothesis, replacement of the terminal sialic acid residue by the action of a sialyltransferase results in the restoration of the normal serum survival time of these proteins (Hickman, 1970). Also, treatment of the asialoglycoproteins with galactose oxidase or β-galactosidase increases their survival time (Morell et al, 1968). These elegant experiments, reviewed by Neufeld and Ashwell (1980) have established the role of β-galactosyl residues and the hepatic lectin in the clearance of desialylated glycoproteins from the serum by the liver. A similar carbohydrate-binding protein is found in chicken liver, but in this case the specificity is for N-acetylglucosamine and in chick serum, asialoglycoproteins have a terminal N-acetylglucosamine group (Lunney and Ashwell, 1976).

3.5.2 Sponge Lectin.

Sponges, although they are the simplest of all multicellular animals, have evolved a complex number of recognition and adhesion systems involving macromolecules at the cell periphery. Early studies by Wilson (1907), Galtsoff (1925) and later by Humphreys (1963) have determined that single cell suspensions obtained from many species of marine sponges either mechanically by squeezing them through a fine mesh cloth or chemically by proteolysis will readily form small aggregates in the presence of Ca^{++}. The earlier workers reported that cell adhesion in sponges was species specific, but the more recent findings indicate that although there is sorting out of cell types in heterotypic aggregates, separation into separate monotypic aggregates does not occur in all cases (Humphreys, 1970; Van de Vyer, 1975). Aggregation is mediated by a soluble, high-molecular weight aggregation factor, and was found to be species specific for several marine sponges (McClay, 1974). The aggregation factor from Microciona parthena has been purified and characterized (Henkart et al, 1973). It is a large glycoprotein complex consisting of approximately equal parts of carbohydrate and protein. Electron

microscopy of these large complex molecules shows a fibrous circle about 800 Å in diameter with 11-15 radiating arms about 1100 Å long, resulting in a sunburst configuration. Removal of calcium dissociates this structure into glycoprotein subunits of 2 x 10^5 molecular weight. It appears that aggregation in Microciona involves at least two distinct steps (Jumblatt, Schlup and Burger, 1980). The first step is a calcium-independent binding of the aggregation factor to the cell surface receptors. This binding can be inhibited by glucuronic acid and destroyed by glucuronidase, indicating that the binding of the aggregation factor depends upon terminal glucuronic acid residues. The second step is the formation of calcium-dependent bonds between the aggregation factors on adjacent cells.

In the sponge Geodia cydonium the adhesion system is somewhat different (reviewed by Müller, 1982). Aggregation in this species also involves a soluble extracellular aggregation factor of high molecular weight. The following components have been shown to be involved in cell aggregation in Geodia by Müller and his coworkers: Firstly, an aggregation factor which is a glycoprotein of approximately 23,000 molecular weight (Müller and Zahn, 1973) and

secondly, a cell surface aggregation receptor of 15,000 molecular weight (Müller et al, 1976). Binding of the aggregation factor to the receptor depends on intact terminal glucuronic acid residues on the receptor. This activity is modulated by an endogenous membrane-associated β-glucuronidase of molecular weight 25,000 which blocks cell aggregation by inactivating the receptor (Müller et al, 1979). A glucuronyl transferase enzyme, also found at the cell surface, activates the receptor by the addition of glucuronic acid, allowing aggregation to proceed. Also involved in this interaction is an anti-aggregation receptor, a glycoprotein of 180,000 molecular weight, which, in its active state, can interfere with the binding between the aggregation factor and the aggregation receptor. The anti-aggregation receptor contains galactose as a major component of its active site and binding to the aggregation receptor is abolished by β-galactosidase. The *in vivo* activity of the anti-aggregation receptor is modulated by a β-galactose specific lectin (Vaith et al, 1979; Müller et al, 1983) of molecular weight 36,500. The binding of the lectin to the anti-aggregation receptor inactivates the latter molecule and allows cells to aggregate via formation of the

aggregation factor-aggregation receptor complex. When lectin is not present the anti-aggregation receptor inactivates the aggregation receptor, allowing cells to separate. In addition, this lectin can also bind to the aggregation factor, and to itself (Conrad et al, 1984). In the opinion of Müller, the lectin is probably not directly involved in aggregation but the interaction of the lectin with the anti-aggregation receptor most likely allows for cell sorting out. It is interesting to note the complexity of cell recognition mechanisms in sponges and also the involvement of lectin-carbohydrate binding reactions in this process.

3.5.3 The Cellular Slime Mold Lectins.

The cellular slime molds are simple eukaryotes which undergo a developmental cycle of alternating single-celled amoebae and multicellular fruiting structures. The single vegetative amoebae exist independently. When food is withdrawn, the cells differentiate into aggregation-competent cells, which then stream together on the substratum and aggregate to form a stable, multicellular plasmodium. Cell movement is directional and comes under the influence

of a chemoattractant. The aggregation phase is complete when all the cells in a given area have concentrated together in one center. Cell movements occur within the center of this aggregate causing it to elongate into a motile slug. The slug differentiates further in the next 12 hours to form a fruiting body consisting of stalk and spore cells.

The mechanisms of adhesion in the cellular slime molds have been extensively studied by immunological (Gerish, 1980) and biochemical (Bartles et al, 1982) methods. Soluble carbohydrate-binding proteins have been isolated from extracts of aggregation-competent cells of Dictyostelium discoideum (Rosen and Barondes, 1972) and Polysphondylium pallidum (Rosen et al, 1974). These lectins are specific for D-galactose and have been affinity purified from columns of Sepharose 4B by elution with D-galactose. In Dictyostelium discoideum, two distinct tetrameric lectins are found. These are known as discoidin I (subunit molecular weight 26,000) and discoidin II (subunit molecular weight 24,000) (Frazier et al, 1975). The lectin from Polysphondylium pallidum, called pallidin, has a subunit molecular weight of 25,000. These lectins appear to be developmentally regulated, since extracts of vegetative cells were

found to contain little or no lectin activity and the activity increased 400-fold in aggregation-competent cells (Rosen et al, 1973). Based on immunofluorescent and immunoferritin labelling studies, about 10% of the protein was found on the cell surface (Chang et al, 1975). Further evidence indicated that cross-linking of the cell surface lectin by binding to antilectin antibodies or glycoconjugate receptors elicited delivery of new lectin molecules to the cell surface (Barondes, 1981).

Endogenous receptors have been shown to be present on the surface of aggregation-competent cells (Reitherman et al, 1975). Bartles and Frazier (1980), using radio-iodinated lectin as a probe, identified two types of receptors on the surface of cells of Dictyostelium discoideum that had been fixed with glutaraldehyde. One type (C sites) bound discoidin I by lectin-carbohydrate interactions and this binding was inhibited by D-galactosides. The number of C sites increased 4- to 6-fold as the cells became aggregation-competent. The second type of receptor bound discoidin I by ionic interactions (I sites) and this binding was detectable only under conditions of low ionic strength (Bartles and Frazier, 1982). Since this binding could not be

inhibited by D-galactose but could be reduced 70-90% by treatment of the fixed cells with chloroform-methanol, the authors suggested that the I sites were ionic lipids. More recently, Barondes and coworkers have described other endogenous receptors for the Dictyostelium discoideum lectins. A polysaccharide synthesized late in development by the prespore cells and localized in the spore coat was found to be an endogenous receptor for discoidin II (Cooper et al, 1983). This polysaccharide contains galactose and N-acetylgalactosamine residues and interacts with the lectin during maturation of the spore coat. The slime coat surrounding the cells in the early aggregate phase appears to contain an endogenous receptor for discoidin I. According to Barondes (1983), this lectin might be involved in the organization and excretion of the extracellular slime trails left on the substratum by migrating amoebae. Discoidin I also promotes attachment and spreading of aggregation-competent cells (Springer et al, 1984). It has been suggested to function in a manner similar to fibronectin.

Barondes (1981) and Bartles et al (1982) have hypothesized that species-specific cell adhesion in slime molds is achieved through interaction between

endogenous lectins and cell surface receptors. In support of such a role for the lectin, mutants of Dictyostelium discoideum have been isolated which synthesize a defective discoidin I molecule lacking the carbohydrate-binding activity (Shinnick and Lerner, 1980). This mutant does not develop beyond the loose aggregate stage. In other studies, antagonists of pallidin such as univalent anti-pallidin antibodies or macromolecules containing galactose residues were found to inhibit aggregation of Polysphondylium pallidum cells. This inhibition, however, occurred only under "permissive" conditions, such as the presence of antimetabolites or in hypertonic medium (Rosen et al, 1977). In contrast to these findings, cell-to-cell adhesion of Dictyostelium discoideum cells cannot be blocked by antidiscoidin antibodies or by galactose-containing molecules. Although many investigators support the notion that lectins are directly involved as ligands in slime mold cell adhesion, a regulatory role for these molecules, similar to the function proposed for the sponge lectin, cannot at the present time be ruled out.

At least three other cell surface proteins distinct from the lectins are also believed to be

involved in slime mold cell adhesion. These include: Firstly, the contact sites B described by Swan et al (1977) which appear to be involved in the EDTA-sensitive cohesion of vegetative cells; secondly, the contact sites A identified by Gerish (1980) which appear to be involved in EDTA-resistant cohesion of aggregation-competent cells; and finally, a plasma membrane glycoprotein, Gp 95, (Steineman and Parish, 1980) which is implicated in EDTA-resistant aggregation of slug cells. Antibodies to any of these cell surface molecules block adhesion of slime mold cells. It is possible that all of these molecules, including the lectins, operate at different developmental times to allow for the aggregation and sorting out of slime mold cells.

3.5.4 Lectins in Fertilization.

In sea urchins, where fertilization has been most extensively studied, sperm-egg adhesion appears to be mediated by the interaction between egg surface glycoconjugates and the acrosomal protein bindin (Glabe and Vacquier, 1978). Bindin (molecular weight 30,500) is liberated by exocytosis from the acrosomal granule upon activation of the sperm. It has been localized by immunohistochemistry using anti-bindin antibodies and is present at the site of

sperm-egg attachment (Moy and Vacquier, 1979). Insoluble bindin particles prepared from sperm membranes agglutinate sea urchin eggs in a species-specific manner (Glabe and Vacquier, 1977). The egg surface receptor for bindin is a high molecular weight polysaccharide component of the egg jelly containing large amounts of fucose and xylose (Glabe and Lennarz, 1981). Bindin can be prepared in a soluble form from the insoluble particles by sonication. The soluble form of this protein agglutinates glutaraldehyde-fixed, trypsinized erythrocytes (Glabe et al, 1982). Bindin-mediated erythrocyte agglutination is inhibited by the same carbohydrates which inhibit egg agglutination; namely, fucoidin, (a sulphated heteropolysaccharide consisting of predominantly L-fucose) and xylan ($\beta(1\text{-}4)$-linked xylose homopolysaccharide). However, inhibition of the haemagglutination reaction is more sensitive than inhibition of the egg agglutination and therefore occurs at lower concentrations of inhibitor (Glabe et al, 1982). This result could reflect a higher affinity of bindin for the egg surface glycoconjugate than for the erythrocyte receptor.

The above authors have implicated lectin-polysaccharide binding in the specific recognition

between sea urchin gametes, the outcome of which is the immobilization of the sperm and the fusion of gametes. Other investigators have described lectin-fucose interactions occurring during fertilization. In the brown algae, Fucus, fertilization involves egg receptors containing sulphated fucose residues (fucoidin) and lectin proteins from sperm (Bolwell et al, 1980). Lectins from sperm and corresponding fucose residues on eggs have also been reported in the tunicate Ciona (Rosati and DeSantis, 1980). It is not known at the present time how widespread lectin-carbohydrate interactions are in fertilization in other organisms.

3.5.5 Galaptins.

The most thoroughly studied group of vertebrate lectins have been called "Galaptins" (Harrison and Chesterton, 1980a). They are soluble, β-galactose binding lectins of low molecular weight (13,000 to 15,000). Their activity is not dependent on the presence of divalent cations. They have been described in a number of adult and embryonic tissues, including chick embryonic muscle, heart, liver and brain (Kobiler and Barondes, 1977; Nowak et al, 1977), calf brain, muscle and liver tissue (Briles et

al, 1979) and neonatal rat lung (Powell and Whitney, 1980). These lectins may represent a group of structurally and functionally related proteins. Some of them show antigenic cross-reactivity (Childs and Feizi, 1979). Many of the galaptins appear to be developmentally regulated since their maximal lectin activity is present at stages of tissue differentiation (Kobiler and Barondes, 1977). An example of this type of galaptin is the erythroid developmental agglutinin (EDA) which has been isolated from adult rabbit bone marrow by Harrison and Chesterton (1980b). In some tissues, galaptins have been localized at the cell surface using immunohistochemical techniques (Gremo et al, 1978). A function has been proposed for this group of lectins in the regulation of cell behaviour during development (Barondes, 1984). However, the actual manner in which the galaptins carry out such a function remains obscure.

3.5.6 Lectins of the Early Chick Embryo.

Early chick embryos at the pregastrula and gastrula stages have been shown to possess surface carbohydrates which can be agglutinated by Ricinus communis agglutinin (RCA), wheat germ agglutinin

(WGA) and Con A (Zalik and Cook, 1976). These results suggest that galactose, mannose and N-acetyl-galactosamine-containing receptors are present on the surface of chick embryonic cells from these early stages. In addition, soluble endogenous lectin can be extracted from homogenates of these embryos (Cook et al, 1979). The lectin agglutinates glutaraldehyde-fixed, trypsinized rabbit erythrocytes and is specific for β-galactosides. This lectin appears to be similar to the galaptins described above although the early chick lectin is present in both a soluble form and a particle-associated form which differ in molecular weight but show similar specificities (Zalik et al, 1983). In a recent study, a lectin with similar properties has been isolated from the area vitellina and area vasculosa of the developing yolk sac in 2-day old chick embryos (Mbamalu, 1985).

Investigators have succeeded in isolating a pure population of extraembryonic endoderm cells from the area opaca of gastrulating embryos (Milos et al, 1979). These cells reorganize in vivo into a single layered epithelium surrounding the yolk sac during gastrulation. Dissociated extraembryonic endoderm cells in tissue culture readily reaggregate to form large aggregates which subsequently hollow out into

vesicles with very thin walls (Milos et al, 1979). The early phase of aggregation of these cells involves both Ca^{++}-independent and Ca^{++}-dependent mechanisms (Milos and Zalik, 1981) but the exact role of Ca^{++} in this system is unclear.

The level of adhesion of extraembryonic cells in rotating culture is significantly decreased in the presence of thiodigalactoside (Cook et al, 1979). Under these conditions, changes in the morphology of the aggregates compared to aggregates formed in control cultures, was also observed. Interestingly, adhesion of these cells has been correlated to the level of lectin activity present in the medium (Milos and Zalik, 1982). Increased levels of lectin activity due presumably to the liberation of soluble lectin by the cultured cells, was associated with decreased cellular adhesion. In other experiments, the presence of soluble blastoderm lectin or TDG added to the cultures also decreased the ability of these endoderm cells to attach to the substratum and to form contacts between adjacent cell (Milos and Zalik, 1981). The morphology of the cells in the presence of lectin or TDG was noticeably different than control cells. Control cells were epithelial in form. In the presence of TDG or lectin, cells

appeared more fibroblast-like. These experiments suggest that lectin secreted by these cells under culture conditions reduces their ability to form attachments either to the substratum or to each other. Recently, these authors have shown that lectin activity can be detected in the fluid in the central cavity of the aggregates. Activity of the lectin is highest at the time when the aggregates are undergoing cavitation (Milos and Zalik, 1986).

Zalik et al (1982) have localized the blastoderm lectin by fluorescent staining of cells in culture with polyvalent antiserum. In common with soluble lectins in other systems, most of the fluorescence was located intercellularly. In migrating cells, retraction fibers were also stained and where cells had detached from the substratum high fluorescence was found in the "footprints". These authors suggest a possible role for this lectin either in the formation of short-term transient adhesive bonds or in the cell attachment and rearrangement taking place during gastrulation. The lectin may act by masking cell surface β-D-galactoside receptors and thus preventing the formation of adhesive contacts between cells. Another possibility is that the lectin may have a

modulating effect on primary adhesive molecules similar to the function proposed for sponge lectins (reviewed above).

Another lectin has been described in unincubated chick eggs (Rutherford and Cook, 1981; 1984). This lectin is located in the vitelline membrane and is specific for mannose-containing polysaccharides. Its relationship, if any, to the β-galactoside binding lectin from chick blastoderms is unknown.

4. PURPOSE OF STUDY

Amphibian embryos during gastrulation undergo extensive cell rearrangements as a result of the migration of cells to new locations and the formation of new adhesive contacts. These morphogenetic movements are most likely mediated by cell surface macromolecules. Galactose-containing receptors for the lectin from Ricinus communis are present on the surface of these cells just prior to gastrulation (Fraser and Zalik, 1976). It is therefore possible that endogenous lectins may be involved in cell surface-mediated phenomena at this critical stage of amphibian development. At the initiation of this study, endogenous lectins had not been described in amphibian embryos, although it was known that chick blastoderms at the time of primitive streak formation

contained an endogenous lectin which bound β-galactose residues (Cook et al, 1979). Therefore, the first approach was to examine embryos of Xenopus laevis for the presence of soluble lectins identifiable by their haemagglutinating ability. Once the presence of these molecules was established, other questions were formulated for study. It was important to determine if the level of lectin activity changed during the time of morphogenetic movements. Levels of activity were compared during cleavage, blastula, gastrula and neurula stages. It was also necessary to determine the saccharide specificity of the lectin being investigated and the optimum conditions necessary for lectin activity. Further work involved the purification and characterization of the Xenopus embryonic lectin, in order to compare this lectin with other similar molecules from different tissues and organisms. Another approach to this problem was to raise antiserum against purified lectin and, using immunohistochemical techniques, to describe the location of the lectin in sectioned embryos of Xenopus laevis.

Results from this study indicate that galactose-binding lectin is present in high levels in Xenopus embryos. The activity of this lectin peaks at the gastrula stage, precisely at the time morphogenetic movements are taking place in the embryo. The lectin is a soluble protein of

65,500 molecular weight and requires calcium for its activity. These properties indicate that the Xenopus embryonic lectin is unlike lectins described in other embryos and other vertebrate tissues. The Xenopus lectin appears to be located extracellularly in vitelline membranes, in the blastocoele cavity and in cleavage furrows in the early embryos which have so far been examined. An attempt to identify the endogenous receptor for this lectin is also presented.

MATERIALS AND METHODS:

1. SOURCES OF LECTIN

1.1 Chemicals:

Human chorionic gonadotropin and β-mercaptoethanol were obtained from Sigma Chemicals. All solutions were prepared in distilled water. In the experiments described here Millipore-filtered water having a resistance of less than 0.05 megohms is referred to as distilled water.

1.2 Xenopus Laevis Embryos:

Xenopus laevis adults were selected from a colony of frogs maintained in the aquatic facility of the Department of Zoology. Individuals in this colony originated either from the South African Snake Farm, Fish Hoek, South Africa, or from tadpoles raised in the Department of Zoology. The frogs were kept in running water at constant temperature (18°C) and under artificial light set at a 12 hour light and 12 hour dark cycle.

To induce spawning and fertilization, healthy mature frogs of both sexes were injected intraperitoneally with human chorionic gonadotropin. Females received 1,000 I.U. in 1 ml of distilled water and males received 500 I.U. in 0.5 ml of distilled water. The frogs were paired

immediately following injection in clean dechlorinated tapwater. Amplexus occurred overnight. The embryos were collected 18 to 20 hours later. Spawning occurred approximately 30% of the time and any clutch which did not contain at least 70% viable embryos was discarded.

Xenopus embryos are surrounded by a dense jelly coat which was removed before further extraction procedures were carried out. The embryos were washed in modified Steinberg's phosphate buffered saline, SPBS, (Hamburger, 1960), which contained NaCl (58 mM), KCl (0.67 mM), $CaNO_3.4H_20$ (0.45 mM), $MgSO_4.7H_20$ (0.83 mM) and phosphate buffer (0.005M) adjusted to pH 7.5. The phosphate buffer stock solution was prepared by adding KH_2PO_4 (0.2M) to 6L of Na_2HPO_4 (0.2M) until the pH reached 7.2. Fifty ml of the stock solution was added to 1L of SPBS. The jelly coats were removed by swirling the embryos in SPBS containing 1% β-mercaptoethanol, pH 8.3 (Johnson, 1976) until visual examination showed disintegration of the jelly coat, usually no longer than 20 to 30 seconds. The embryos were immediately rinsed in 100 ml of SPBS, swirled briefly and the mixture was decanted. This washing was repeated at least ten times to ensure that no residual mercaptoethanol remained since it adversely affects the

further development of the embryo. With adequate washing, embryos left in dechlorinated tapwater develop normally.

The embryos were staged according to Niewkoop and Faber (1967). Only viable, healthy embryos at the proper stage were used for each experiment. In general, four groups of embryonic stages were used: cleavage, stages 3-5; blastula, stages 7-9; gastrula, stages 10 and 11; and neurula, stages 20-22. These stages are shown in Figure 1. Sometimes, experiments were performed with embryos at stages other than those described here. In such cases the exact stage will be noted in the text. In total, approximately 48,000 embryos were used to provide lectin extracts for the experiments described here.

2. LECTIN EXTRACTION

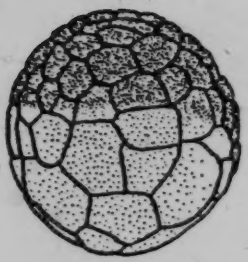
2.1 Chemicals:

Lactose, phenylmethylsulphonylfluoride (PMSF), bovine serum albumin (BSA), crystallized, catalogue number A4378, and trypsin, Type III, 10,000-13,000 BAEE units per mg of protein, were all obtained from Sigma Chemicals. Glycine and microtiter plates (Cooke Engineering) were purchased from Fisher Scientific. Glutaraldehyde, 70% concentrated, was from Ladd Chemicals. Nitex cloth was from B and S.M. Thompson and Co., Montreal.

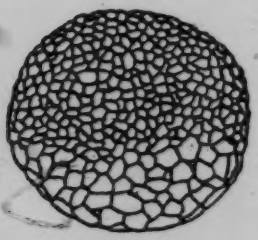
FIGURE 1: Early embryonic stages of Xenopus laevis used in these experiments. Stages 3 and 4 are cleavage, 7 and 8 are blastula, 10 and 11 are gastrula and 20 and 22 are neurula. An; Animal Pole. Veg; Vegetal Pole. Ventr; Ventral Surface. Dors; Dorsal Surface. The diagrams are adapted from Niewkoop and Faber (1967).

St.3 An.

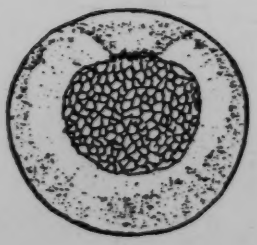
St.4 An.



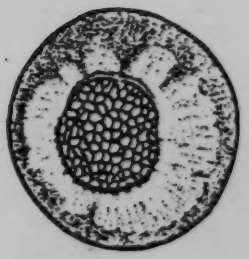
St.7 Ventr.



St.8 Dors.

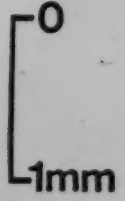


St.10 Veg.



St.11 Veg.

St.20 Dors.



St. 22 Dors.

2.2 Preparation of Soluble Lectin Extracts:

Selected embryos were homogenized in a lectin extraction solution (LES), consisting of SPBS with NaN_3 (0.02%), lactose (0.3M), and PMSF (0.25 mM), pH 7.5, at a ratio of 20 µl per embryo. The addition of lactose to the extraction solution increased the level of haemagglutinating activity of the extract by approximately 3-fold and was therefore included in all extractions. The homogenate was passed through Nitex cloth (44 µm) to filter out the vitelline membranes. At this point the homogenate could be stored frozen for a period of several months at -20°C. Following defrosting the soluble lectin was extracted at 4°C for two hours with continual stirring. The mixture was then centrifuged at 84,000 x g for 60 minutes in either a Beckman L-60 ultracentrifuge using rotor SW 28, or an IEC M-25 centrifuge using rotor 896. The pellet was stored at -20°C and the supernatant was removed and dialysed in Spectrapor membrane dialysis tubing (molecular weight cut-off 6,000) against five changes of 40 x volume of SPBS. The resulting solution was used directly for the assessment of lectin activity.

Soluble lectin was occasionally obtained from minced ovarian tissue. In these experiments ovaries were dissected from mature female frogs. The tissue was minced finely with scissors, removing as much connective tissue

as possible, and then added to LES (500 µl per gram of tissue). The mixture was homogenzed in a Potter-Elvehjem tissue homogenizer and more LES added as required to produce a suspension (up to 5 x volume). The lectin was extracted as described for embryonic homogenates except that the extraction of the ovarian tissue was allowed to continue for 24 hours.

2.3 Measurement of Lectin Activity:

Lectin activity was measured by haemagglutination assays performed in microtiter V plates (Cooke Engineering) at room temperature. Fifty µl of lectin extract was serially diluted in 50 µl SPBS, followed by the addition of 25 µl 1% BSA in PBS (0.15M NaCl in 0.005M phosphate buffer, pH 7.20. Finally 25 µl of a 4% solution of rabbit erythrocytes, prepared as described below, was added. The plates were shaken briefly and allowed to settle for one hour. Positive agglutination was assessed visually by the presence of a dense mat of erythrocytes covering the bottom of the well. Control wells containing 50 µl of saline and having no haemagglutinating activity showed a discrete button of erythrocytes at the well bottom (see Figure 4). In accordance with other authors (Dysart and Edwards, 1977; Nowak et al, 1976), lectin activity is expressed as the number of haemagglutinating

units (HU) present, which equals the reciprocal of the titer or the highest dilution giving positive agglutination. All titrations were performed in duplicate and the average value was recorded.

The rabbit erythrocytes used in the haemagglutination assays were prepared from whole blood collected into sterile Alsever's solution (10.5 g dextrose, 4 g sodium citrate, 0.275 g citric acid and 2.1 g sodium chloride, pH 6.1). Ten ml of rabbit blood was dispensed into 20 ml Alsever's solution, and further treated by one of the following two methods. Initially, trypsin-treated, glutaraldehyde-fixed erythrocytes were prepared according to the method of Nowak et al (1977) as shown in the flow chart in Figure 2. Because some preparations of erythrocytes treated in this manner gave inconsistent results, later assays were conducted using erythrocytes preserved by an alternate method involving 0.02% sodium azide and 0.25 mM PMSF as described by Zalik et al (1983) and shown in the flow chart in Figure 3. This latter method resulted in cells which retained their activity for about ten days, compared to six weeks for the glutaraldehyde-fixed cells, but more consistent titers were obtained between different preparations of erythrocytes.

FIGURE 2: A flow-chart outlining the procedure used to prepare trypsin-treated glutaraldehyde-fixed rabbit erythrocytes (Nowak et al, 1977). These erythrocytes were used in the haemagglutination assay.

10 ML RABBIT BLOOD IN 20 ML ALSEVER'S SOLUTION

↓ CENTRIFUGE 900X G, 10 MIN.

WASH 4x WITH 5 VOLUMES OF 0.15 M NaCl in 0.005 M $NaKPO_4$ BUFFER, pH 7.1.

↓ MEASURE VOLUME OF PACKED CELLS

INCUBATE IN TRYPSIN (0.04 MG/ML PACKED CELLS) DISSOLVED IN 0.05 M NaCl in 0.1 M $NaKPO_4$ BUFFER, pH 7.1, 37°C. 1HR.

↓ CENTRIFUGE 900 XG, 10 MIN.

WASH 4x IN 5 VOLUMES 0.15 M NaCl IN 0.005 M $NaKPO_4$ BUFFER, pH 7.1.

↓ MEASURE VOLUME OF PACKED CELLS

ADD 5x VOLUME 1% GLUTARALDEHYDE IN 0.075 M NaCl IN 0.075 M $NaKPO_4$ BUFFER, pH 7.1, ON SHAKER, 1HR., R.T.

↓ CENTRIFUGE, MEASURE VOLUME OF PACKED CELLS

ADD 10x VOLUME ICE-COLD GLYCINE, 0.1 M, IN 0.075 M NaCl IN 0.075 M $NaKPO_4$ BUFFER, pH 7.1, WASH 3x PLACING ON SHAKER 5 MIN BETWEEN EACH WASH

WASH 2x WITH 15x QUANTITY OF 0.15 M NaCl IN 0.005 M $NaKPO_4$ BUFFER, pH 7.1.

↓ MEASURE VOLUME OF PACKED CELLS.

ADD 0.075 M NaCl IN 0.075 M $NaKPO_4$ BUFFER, pH 7.1, TO MAKE A 10% SOLUTION. STORE AT 4°C.

FIGURE 3: A flow-chart outlining the procedure used to prepare trypsin-treated rabbit erythrocytes, used in the haemagglutination assay (Zalik et al, 1983).

10 ML RABBIT BLOOD IN 20 ML ALSEVER'S SOLUTION

↓ CENTRIFUGE 900x G, 10 MIN.

WASH 4x WITH 5x VOLUME OF 0.15 M NaCl in 0.005 M $NaKPO_4$ BUFFER, pH 7.1.

↓ MEASURE VOLUME OF PACKED CELLS

INCUBATE IN TRYPSIN (0.04 MG/ML PACKED CELLS) DISSOLVED IN 0.05 M NaCl in 0.1 M $NaKPO_4$ BUFFER, pH 7.1, 37°C. 1HR.

↓ CENTRIFUGE 900x G, 10 MIN.

WASH 4x IN 5x VOLUME 0.075 M NaCl IN 0.075 M $NaKPO_4$ BUFFER CONTAINING 0.02% NaN_3 AND 0.25 mM PMSF, pH 7.2

↓ CENTRIFUGE, MEASURE VOLUME OF PACKED CELLS

ADD 0.075 M NaCl IN 0.075 M $NaKPO_4$ BUFFER CONTAINING 0.02% NaN_3 AND 0.25 mM PMSF, pH 7.2, TO MAKE A 10% SOLUTION. STORE AT 4°C.

The concentration of protein present in the crude supernatants was also measured. The method used the BioRad protein assay kit as described in the BioRad Technical Bulletin, number 1051, and adapted from the method of Bradford (1976). The standard used was bovine plasma gamma globulin. For each test, a series of six standards were prepared ranging in concentration from 0.2 to 1.4 mg/ml for the standard assay or 1 to 25 µg/ml for the microassay. Dye reagent was added to standard and sample tubes and after gently vortexing the mixtures, the optical density at 595 nm was read in a Beckman DU-8 spectrophotometer. A standard curve was plotted and used to determine protein concentrations of the extracts.

For each separate clutch of embryos used for lectin extraction, the following parameters were measured and recorded: stage, number of embryos used, total lectin activity in the extract in HU, average HU/embryo, total protein extracted, protein/embryo and specific activity (HU/mg protein) of the extract.

2.4 Measurement of Lectin Activity in the Blastocoele Cavity:

Fluid from the blastocoele cavity was obtained from two stage 8 embryos from which the jelly coats had been

manually removed. Each embryo was immobilized in one well of a microtiter plate with the animal pole uppermost. The needle of a Hamilton microsyringe (series 7,000) 5 µl capacity, was carefully inserted through the upper layer of cells and into the blastocoele cavity. Approximately 0.05 µl of fluid was removed from the cavity of each embryo and mixed with 75 µl SPBS. This mixture was used for the haemagglutination assay.

3. DETERMINATION OF SACCHARIDE SPECIFICITY

3.1 Chemicals:

All sugars were obtained from Sigma Chemicals. Immunoadsorbents were kindly donated by Dr. R. Lemieux of the Chemistry Department, University of Alberta. Ethylenediaminetetraacetic acid (EDTA) was from Baker Chemicals and ethylene bis(oxyethylene nitrilo)tetraacetic acid (EGTA) was from Eastman Kodak Co.

3.2 Saccharide Inhibition Tests:

In order to determine the specificity of the lectin for certain monosaccharides and disaccharides, saccharide inhibition tests were performed using a modification of the haemagglutination assay described in Section 2.3. For

each test, 25 µl saccharide solution (25 mM dissolved in SPBS) was serially diluted in SPBS, followed by the addition of 25 µl lectin extract containing 4 HU, 25 µl BSA (1% in PBS) and 25 µl rabbit erythrocytes. The lowest saccharide concentration which inhibited 4 HU was recorded. The following saccharides were used: lactose (β-D-Gal(1-4)Glc), thiodigalactoside (β-D-Gal(1-1)β-D-thiogal), melibiose (α-D-Gal(1-6)Glc), methyl α-D-galactopyranoside, methyl β-D-galactopyranoside, fucose, methyl α-D-mannopyranoside, N-acetyl-D-galactosamine and N-acetyl-D-glucosamine.

This test was also used to determine the effect of the chelating agents EDTA and EGTA on the lectin activity. In this case, the saline used throughout was Ca^{++} and Mg^{++}-free SPBS. The lowest concentration of chelating agent required for inhibition of 4 HU of lectin activity was recorded.

Immunoadsorbents (IA) were used to further define the saccharide specificity of this lectin. IA are prepared by covalently coupling synthetic mono- or disaccharides to an insoluble matrix of crystalline silica. The average concentration of bound sugars is 0.3 to 0.5 moles per gram of immunoadsorbent (ChemBioMed, U. of Alberta). IA bearing the following attached sugars were used in these experiments (-R represents the solid matrix): DGlcα1-R,

DGalα1-R, DGalβ1-R, DGalNAcα1-R, lactose (DGal(β1-4)DGalβ1-R), DGal (α1-3)DGalβ1-R, DGal(α1-4)DGalβ1-R, DGal(β1-3)DGalNAcα1-R), DGal(β1-3)DGlcNAcα1-R, DGal(β1-3)DGlcNAcβ1-R, DGal(β1-4)DGlcNAcβ1-R, (Lemieux, 1978). Crude lectin extract was incubated with IA at a concentration of 54,000 HU per mg IA in a rotating shaker (200 r.p.m.) at 4°C for two hours. This relative concentration of DGal(α1-3)DGalβ1-R bound essentially all of the lectin activity in initial experiments and therefore all other IA's were used at the same concentration. After incubation, the supernatant was removed and tested for agglutinating activity. Inhibition values represent the decrease in the activity of the supernatant compared to the initial extract.

4. LECTIN PURIFICATION

Attempts were made to purify the lectin by taking advantage of the binding between the lectin molecule and galactose-containing saccharides. The general procedure used for purification involved exposing supernatants of crude lectin to matrices bearing terminal galactose residues and removing material bound to the matrix by washing extensively with saline containing lactose. The details

of three separate methods carried out during this study are described here.

4.1 Chemicals:

Sepharose 4B was obtained from Pharmacia Fine Chemicals; Selectin-4 from Pierce Chemicals. Para-aminophenyl-β-D-lactoside was obtained from Vega Fox Biochemicals; cyanogen bromide from Aldrich Chemicals; succinic anhydride from Terochem. Polyethylene glycol (Carbowax, PEG 20,000) was purchased from Fisher Scientific.

4.2 Batch Adsorption to αGal-R-Immunoadsorbent:

The αGal-IA was added to the crude lectin extract at a ratio of 10 mg IA per ml of extract (containing an average of 261,590 HU). The mixture was incubated at 4°C from one to two hours on a rotating shaker set at 200 r.p.m. The IA was allowed to settle and the supernatant was removed. The activity of this residual supernatant was measured and compared to the activity of the original extract to monitor the amount of lectin bound. The beads were then washed with several volumes of SPBS and bound material was eluted by a brief washing with a 2 ml volume of NH_4OH (1%) in 0.07 M NaCl with or without the addition of 0.3 M lactose. Similar recoveries were

obtained when elution was carried out in the presence or absence of lactose. To ensure that the maximum amount of lectin was recovered, lactose was routinely included in the elution medium. The eluant was immediately dialysed against SPBS and the agglutinating activity was measured. This was the most frequently used method for lectin purification.

4.3 Affinity Chromatography using APL-Sepharose:

The lectin could also be purified on a column containing Sepharose-4B coupled to para-aminophenyl-β-D-lactoside (APL-Sepharose). This matrix was prepared by the method of Nowak et al (1977). The procedure involves, firstly, activation of the matrix by cyanogen bromide treatment and addition of hexanediamine which acts as a spacer arm. Secondly, the spacer arm is reacted with succinic anhydride to form a derivative to which amino groups can be coupled. Finally, para-aminophenyl-β-D-lactoside is covalently attached in the presence of 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide at acid pH. Residual active groups are blocked by exposing the gel to ethanolamine at alkaline pH. Excess uncoupled ligand is removed by several alternate washings with 0.1 M bicarbonate buffer, pH 9.1 and 0.1 M acetate buffer, pH 4, each containing NaCl (1M).

The APL-Sepharose matrix was suspended in SPBS containing NaN_3 (0.02%), poured into a column (0.5 x 20 cm) and packed at a flow rate of 10 mls per hour.

For lectin purification, crude extract with an average activity of 2 x 10^6 HU was added to the column and eluted with SPBS (70 ml), followed by SPBS containing 0.3 M sucrose (35 ml) and finally SPBS with 0.3 M lactose, until the optical density baseline was re-established. The flow rate was 4 ml/hr and 3.5 ml fractions were collected. Lectin activity in the first 20 tubes was measured directly while tubes containing sucrose or lactose were dialysed against SPBS before being tested for activity.

4.4 Affinity Chromatography Using Selectin-4:

Selectin-4 consists of cross-linked 4% agarose beads containing immobilized melibiose, (DGal(α1-6)DGlc). An attempt was made to purify the Xenopus lectin according to the method reported by Roberson and Barondes (1982). Crude extract (2 ml) containing 1-5 x 10^6 HU was loaded onto a column (0.5 x 20 cm) that had previously been packed with Selectin-4 using SPBS at a flow rate of 10 mls per hour. The column was washed consecutively with SPBS (100 ml), SPBS with 0.3 M sucrose (35 ml) and SPBS with 0.3 M melibiose or lactose. Fractions were collected and dialysed as above.

4.5 Chloroform-Methanol Treatment:

Crude soluble lectin extracts from Xenopus embryos prepared as described above are cloudy because they contain variable amounts of suspended lipid-like material. In contrast, purified extracts appear clear but nevertheless may retain some of this lipid material associated with the lectin molecules. In an attempt to remove this contamination, crude lectin extracts were treated with an equal volume of chloroform-methanol (2:1 v/v) by the method of Hamaguchi and Cleve (1972). The mixture was stirred for 15 minutes at room temperature and centrifuged at 900 x g for 10 minutes. The upper aqueous layer was dialysed against SPBS and lectin activity measured. Essentially all of the activity was recovered in the aqueous layer after this procedure. The chloroform-methanol treated crude extract was purified as described above. In some experiments, the lectin preparation was treated with chloroform-methanol after purification and the aqueous layer containing the lectin activity was dialysed against SPBS before use.

4.6 Concentration:

The crude or purified lectin extracts in some experiments were concentrated. The solution containing

the lectin was sometimes placed in dialysis tubing and covered with solid PEG. This procedure resulted in a 5-10 x increase in protein concentration although lectin activity was not always increased proportionally. More frequently, purified extracts were lyophilized in a freeze dryer (Virtis 10-145-BA). In some experiments, chloroform-methanol treated purified extract was precipitated by the addition of 7 volumes cold 98% ethanol according to the procedure of Hamaguchi and Cleve (1972), centrifuged at 900 x g in a clinical centrifuge for 20 minutes and the pellet redissolved in SPBS.

5. GEL FILTRATION CHROMATOGRAPHY

5.1 Chemicals:

Sephacryl S-300 Superfine and Sephacryl S-200 Superfine were obtained from Pharmacia Fine Chemicals. The molecular weight standards were all from the Pharmacia Fine Chemicals' low molecular weight gel filtration calibration kit. Octylglucoside was purchased from Sigma Chemicals and urea was from BioRad. Triton X-100 was from Rohm and Haas.

5.2 Gel Filtration Chromatography of Xenopus Lectin:

The columns were packed with Sephacryl S-200 or Sephacryl S-300 according to the manufacturer's instructions. The preswollen matrix was washed well with SPBS to remove the fines and packed into a column (2.6 x 40 cm) at 4°C and a flow rate of 50 ml/hr. All buffers used contained NaN_3 (0.02%) as a preservative.

Solutions containing crude or purified lectin were chromatographed at a flow rate of 15 ml/hr. Initially the elution buffer used was SPBS. Under these conditions, it was found that the lectin had a tendency to elute as a continuum of low to high molecular weight rather than as a discrete peak. For this reason the columns were subsequently eluted with one of the following buffers: SPBS + octylglucoside (0.25%), SPBS made 1 M with respect to NaCl, SPBS + urea (2 M), SPBS + lactose (0.1 M). All buffers were adjusted to pH 7.3. Fractions (3.5 ml) were collected and the agglutinating activity of each fraction was determined separately. When the fractions contained lactose, they were dialysed against SPBS before being used for the haemagglutination tests. With each of the buffers used, the column was calibrated with the following molecular weight standards: thyroglobulin (660,000), ferritin (440,000), catalase (232,000), bovine serum albumin (67,000), ovalbumin (45,000), chymotrypsinogen (25,000)

and ribonuclease (13,700). The partition coefficient (K_{av}) was determined for each standard, using the equation:

$$K_{av}=(v_e-v_o)/(v_t-v_o),$$

where v_e is the elution volume,

v_o the void volume, and

v_t the total bed volume.

The K_{av} values were plotted against molecular weight and used to assign apparent molecular weights to the lectin fractions.

6. SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

6.1 Chemicals:

Acrylamide, Bis (N,N-methylene-bis-acrylamide), sodium dodecylsulphate and bromophenol blue were purchased from BioRad. TEMED (N,N,N,N-tetramethyl-ethylenediamine) was from Eastman Kodak and TRIS (hydroxymethylaminomethane) was from Fisher. Molecular weight standards were obtained premixed in kits from BioRad or Pharmacia Fine Chemicals. Coomassie brilliant blue was purchased from Sigma Chemicals or BioRad, silver nitrate and bromophenol blue from Fisher Scientific and basic fuchsin from BioRad.

6.2 Procedure:

Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecylsulphate (SDS) was carried out according to the procedure of Laemmli (1970) using 9 to 11% flat bed polyacrylamide gels run on an LKB 2117 multiphor apparatus. The gels were prepared in a casting assembly supplied by LKB. The solution for a 10% gel was prepared from a stock acrylamide solution (60 g acrylamide, 1.6 g bisacrylamide and distilled water to 100 ml) as follows: 13.3 ml of stock solution was mixed with 6.7 ml distilled water. Of this, 16 ml was added to 14 ml distilled water, swirled and degassed. Next was added 15 ml ammonium persulphate solution (2 mg/ml in 1% SDS) and 15 ml TEMED solution (0.13 ml TEMED and 18.2 g TRIS made up to 100 ml with distilled water, pH 8.3). This solution was immediately poured between the two glass plates of the assembly and allowed to stand overnight at room temperature before use.

Samples for PAGE were dialysed extensively against distilled water, lyophilized and solubilized in sample buffer which was composed of TRIS (0.05 M), SDS (2% w/v), β-mercaptoethanol (5% v/v), and urea (8 M), pH 8.5. The final protein concentration was typically 1-4 µg/µl. Samples were heated at 60°C for 30 minutes and 15 µl

volumes applied to the gels. In some cases lectin samples that had not been previously treated with chloroform-methanol were subjected to more drastic procedures to achieve complete dissociation. The methods used were those of Kawasaki and Ashwell (1976) for the reduction of the N-acetylglucosamine binding protein of the avian liver. Purified lectin was incubated for three days at 48°C in SDS (1%) in distilled water. Subsequently, β-mercaptoethanol (10% v/v) and urea (5 M) were added and the sample was incubated for an additional 3 hours at 48°C. Alternatively, the lectin was dissolved in Na_2CO_3 (1%) containing SDS (1%) and β-mercaptoethanol (10%) and dialysed against SDS (0.1%) in $NaKPO_4$ buffer (0.01 M) for 24 hours (Glossman and Neville, 1971). Neither of the above procedures changed the resulting banding patterns of the samples.

The following proteins were used as molecular weight standards: phosphorylase B (97,000), bovine serum albumin (67,000), ovalbumin (45,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,000) or chymotrypsinogen (25,000) and lysozyme (14,000) or ribonuclease (13,700). Mixtures of these standards or individual standards were diluted in sample buffer at a concentration of 0.01 μg/μl and heated at 60°C for 30 minutes as above.

The PAGE gel was prerun at 300 V and 300 pulses per second (pps) for 3 hours or until a brown band of impurities had travelled halfway across the gel. Samples, 15 µl each, were added to the wells and the power was started at 50 V and 50 pps and increased at half hour intervals until 300 V and 300 pps was reached. The temperature was maintained at 5°C throughout the run. The running buffer contained TRIS (15.17 g), glycine (72.08 g) and SDS (5 g) in 5 L distilled water, pH 8.3. Electrophoresis was terminated when the marker bromophenol blue added to the standard wells had transversed the gel, usually in 4 to 5 hours.

The gels were stained in either Coomassie brilliant blue (Fairbanks, Steck and Wallach, 1971) or with silver stain (Wray, Boulikas, Wray and Hancock, 1981). Staining for carbohydrate was performed by the periodic acid-Schiff method of Fairbanks, Steck and Wallach (1971). These procedures are as follows:

Coomassie Blue Protein Stain:

- Soak gel overnight in a filtered solution of Coomassie brilliant blue R-250 (0.04%), isopropyl alcohol (25%) and glacial acetic acid (10%) in distilled water.
- Destain in several changes of glacial acetic acid (10%) and isopropyl alcohol (10%) in distilled water.

Silver Stain:

- Soak gel overnight in methanol (50%) in distilled water containing formalin (0.1 ml/L).
- Soak in 2 changes of distilled water for 30 minutes each.
- Soak in methanol (50%) in distilled water, 0.5-2 hours.
- Stain 15 minutes in silver reagent [mix 42 ml NaOH (0.36%) with 2.8 ml concentrated ammonium hydroxide. Add, with stirring, 1.6 g $AgNO_3$ dissolved in 8 ml distilled water, make up to 200 ml with distilled water, use within 5 minutes]. Use constant gentle agitation.
- Wash in distilled water, 5 minutes.
- Wash in two changes of developer [2.5 ml citric acid (1%) and 0.25 ml formaldehyde (38%) made up to 500 ml with distilled water, prepared fresh], 10 minutes first wash, followed by a second wash until the desired degree of staining is reached.
- Rinse in distilled water for 30 minutes.
- Rinse in methanol (50%) for 1 hour.
- Store in distilled water.
- If overstained, the gels were destained by soaking in Kodak Rapid Fix until bands were of desired intensity, followed by washing in Kodak Hypoclear for one hour. This was followed by rinsing in methanol (50%) for one hour and storing in distilled water.

Schiff Periodic Acid Stain for Carbohydrates (PAS)

- Fix gels overnight in isopropyl alcohol (25%) and acetic acid (10%).
- Soak in isopropyl alcohol (10%) and acetic acid (10%), 6-9 hours.
- Soak gels in acetic acid (10%) overnight and again in fresh solution for 1-2 hours.
- Soak gels in periodic acid (0.5%) for 2 hours.
- Wash in sodium arsenite (0.5%) and acetic acid (5%) for 30-60 minutes and again in sodium arsenite (0.1%) and acetic acid (5%) for 20 minutes. Repeat twice. Wash in acetic acid (5%) 10-20 minutes.
- Soak gel overnight in Schiff reagent, prepared according to Maddy (1976) by dissolving basic fuchsin (1.5 g) in 500 ml boiling water. The solution was filtered at 55°C, cooled to 40°C and 25 ml HCl (2 M) was added followed by 3.75 g sodium metabisulphite. After 16 hours at 4°C, activated charcoal (1.25 g) was added and the mixture shaken and filtered. The reagent was stored at 4°C.

For staining, the gels were placed into Pyrex glass dishes except for Coomassie blue staining which could also be performed in a stainless steel staining tray obtained from LKB. Throughout the procedure the dishes were constantly agitated at 40 r.p.m. Initially the gels

were stained in Coomassie blue or silver stain alone, but later in this study gels were stained in Coomassie blue, destained in methanol (50%) overnight and restained in silver the next day.

The length of the gel was measured both before (L_i) and after (L_f) staining. The distances travelled from the origin by the standard proteins were measured after staining and the mobility values (M_r) calculated using the equation

$$M_r = \frac{\text{distance travelled by protein}}{\text{distance travelled by tracking dye}} \times \frac{L_i}{L_f} .$$

A standard curve was determined by linear regression analysis of M_r values plotted against Log_{10} molecular weight. This curve was then used to assess the approximate molecular weight of the sample bands.

Gels were usually photographed to provide a permanent record. Photographs were taken with a Nikon FE camera using Kodak Tech-Pan or Pan-X film.

7. ISOELECTRIC FOCUSSING

7.1 Chemicals:

The standards were obtained in a premixed form from Pharmacia Fine Chemicals' calibration kit for

isoelectric focussing. Ampholines, pH 3.5-10, were purchased from LKB. Riboflavin was from Eastman Kodak and Cambrelle paper from Industrial Chemical Company, U.K.

7.2 Procedures:

An LKB Multiphor apparatus, model 2117, was used for isoelectric focussing. The gels were prepared using a stock acrylamide solution containing acrylamide (28 g) and bis-acrylamide (0.7 g) made up to 100 ml with distilled water, and a catalyst stock solution containing riboflavin (7 mg) and TEMED (0.7 ml) made up to 100 ml with distilled water. Both solutions were stored in brown bottles at 4°C and used over a period of several months. To prepare the gel, 14.7 ml acrylamide stock solution and 1.8 ml ampholine mixture (pH 3.5-10) were added to 41.4 ml distilled water and the mixture was degassed under vacuum for approximately 10 minutes. Then, 4.2 ml of the catalyst solution was added, the solution was mixed and poured into a casting assembly. The gel was allowed to polymerize overnight in the presence of a fluorescent light.

Purified, lyophilized lectin (35-80 µg) and standards (25-50 µg) were dissolved in 1% glycine to increase their solubility (LKB Application Note 250). The standards included amyloglucosidase (pI 3.5), soybean trypsin

inhibitor (pI 4.55), β-lactoglobulin A (pI 5.2), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.55), horse myoglobin (pI 6.85 and 7.35), lentil lectin (pI 8.15, 8.45 and 8.65) and trypsinogen (pI 9.3). Samples and standards (100 µl) were applied to small rectangular pieces of Cambrelle paper placed approximately halfway down the gel surface. The gel was focussed according to the method of Cook (1976) using a LKB 3371E DC power supply. The power was initially set at 200 volts and increased by 200 volts until a maximum of 1200 volts was reached. The current was maintained at 10 mA throughout the experiment. Focussing was allowed to continue either for five hours or overnight. Following the run, the pH was measured with a surface electrode and the power was again turned on for 30 minutes to sharpen the bands. For staining in Coomassie blue, the gels were first fixed in trichloroacetic acid (12.5% w/v) in distilled water for 18 hours. After several washes in distilled water the gels were immersed in staining solution consisting of 250 ml Coomassie blue stock solution (0.5 g Coomassie brilliant blue R-250 dissolved in 500 ml 95% ethanol and filtered), 950 ml 95% ethanol, 480 ml glacial acetic acid and 1200 ml distilled water. The gels were stained overnight and background staining was removed by washing in destaining solution (200 ml 95% ethanol, 80 ml glacial

acetic acid, 520 ml distilled water and 200 ml glycerol). Sometimes these gels were stained in periodic acid Schiff stain and photographed as described for PAGE gels.

After staining, the distances travelled by the bands were measured and a standard curve drawn by plotting either distances against pI values for the standard proteins, or distances against pH values measured by the surface electrode. The pI of the protein sample was estimated by comparison to the standard curves.

8. AMINO ACID ANALYSIS

The amino acid composition of purified lectin protein was analysed by the Department of Biochemistry, University of Alberta, using a Durram D-500 Amino Acid analyser. An αGal-IA purified, chloroform-methanol treated sample from gastrula embryos was analysed after hydrolysis in 6N HCl for 24 hours.

9. DETECTION OF A POSSIBLE ENDOGENOUS LECTIN RECEPTOR

The procedure previously described in Section 2.2 for obtaining soluble lectin extracts produced a pellet after centrifugation of the homogenate at 84,000 x g for 60 minutes. This pellet which in theory should contain

membranes and other cellular organelles, was further examined for the presence of a fraction containing a possible membrane-associated lectin receptor. The pellets from three clutches of gastrula embryos were combined and resuspended in SPBS containing Triton X-100 (1%). This material was stirred for 24 hours at 4°C and centrifuged at 84,000 x g for 60 minutes. The supernatant was chromatographed on a column containing Sephacryl S-300 which had been washed and calibrated with SPBS containing Triton X-100 (0.05%), (T-SPBS). A 2 ml volume was added to the column and 3.5 ml fractions were eluted in T-SPBS. Each fraction was extensively dialysed against SPBS and tested for the ability to inhibit the agglutinating activity of a lectin extract having a titer of 128 HU. For these tests, 25 µl of each fraction of the solubilized membrane pellet was added to 50 µl of lectin extract which had been serially diluted in a microtiter plate. BSA (1%), 25 µl, and 25 µl of a 4% erythrocyte suspension were then added and the plate treated as described for the saccharide inhibition tests. Inhibitory activity is expressed as the percent reduction in the haemagglutinating activity of the lectin in the presence of each fraction, as compared to the haemagglutinating activity in the presence of SPBS.

10. LOCALIZATION OF THE LECTIN

10.1 Chemicals:

Freund's complete and incomplete adjuvant was from Difco Laboratories. Goat anti-rabbit immunoglobulin coupled to horseradish peroxidase (GAR-HRP), GAR-HRP color development kit and nitrocellulose sheets were purchased from BioRad. Napthol Blue Black was from Eastman Kodak. Fluorescein isothiocyanate-labelled (FITC) goat anti-rabbit IgG was from Miles-Yeda. 1,4-Diazobicyclo [2-2-2] Octane (Dabco) was from Sigma. OCT compound was from Miles Laboratories.

10.2 Antibody Preparation:

A male, six-month old rabbit weighing 4.69 kg was used for production of the antiserum. The lectin sample (90 µg in 1100 µl SPBS) was emulsified in complete Freund's adjuvant (1:1 v/v). Subcutaneous injections were given to the rabbit at 14 sites. Pre-immune serum was obtained from the animal before injection. Six weeks after the initial injection, the rabbit was boosted with 10 µg lectin emulsified in incomplete Freund's adjuvant (1:1 v/v). Similar boosting was repeated 10 days later with 17 µg lectin mixed 1:1 v/v with incomplete Freund's adjuvant. The rabbit was bled on three separate occasions

to provide immune serum. The first bleeding was done five weeks after the intial injection and the rabbit was bled again one week after each of the booster injections. Pre-immune and immune serum were processed in the same way. The whole blood was left overnight at 4°C and cleared by centrifuging twice at 900 x g for 30 minutes in a clinical centrifuge. The supernatant was spun at 84,000 x g for 30 minutes in an IEC M25 centrifuge at 4°C. The serum was divided into 500 µl aliquots, labelled and frozen at -70°C.

10.3 The Characterization of the Antiserum:

10.3.1 Agar Diffusion Tests.

The agar diffusion test was one method used to test the presence and the specificity of the antibody in the antiserum prepared as described above. Agar plates were prepared from agarose (0.3%) in borate buffered saline (BBS), which consisted of NaN_3 (0.02%) and lactose (0.3 M) in 95 parts NaCl (0.85%) and 5 parts borate buffer. The borate buffer contained boric acid (6.184 g), Na tetraborate (9.536 g), NaCl (4.354 g) and distilled water to 1 L, pH 8-8.5. The agarose mixture was autoclaved for 3 minutes and 0.5 ml was dispensed into tissue

culture dishes (10 x 35 mm) which were then allowed to dry without lids in the oven at 75°C for 45 minutes. Following this, 0.6% agarose (2 mls) prepared as above was layered over the dry agar and left at room temperature to set. Six peripheral holes and one central hole were punched out of the agar when thoroughly set.

Crude or purified lectin was introduced into the center well and antiserum diluted in BBS was added to the outer wells, or alternatively, antiserum was added to the center well and lectin diluted in SPBS was added to the outer wells. After sitting overnight the plates were scored for the presence of bands. Bands were in some cases enhanced according to the technique described by Renn and Evans (1975). The agar was covered with freshly prepared phosphotungstic acid (0.5%) for 15 minutes followed by washing with NaCl (0.85%) containing NaN_3 (0.05%).

10.3.2 Inhibition of Agglutination.

The immune serum was tested to determine if it could inhibit the lectin-induced agglutination of erythrocytes. Crude lectin extract from blastula embryos was mixed in test tubes (1:1 v/v) with either SPBS, increasing dilutions of control serum or

increasing dilutions of anti-lectin antiserum. The tubes were shaken briefly and allowed to incubate overnight at 4°C. The tubes were centrifuged in a Beckman microfuge at 7,000 x g for 10 minutes, and the supernatant was tested for haemagglutinating activity as previously described (Section 9).

10.3.3 Protein Blotting and Immunoblotting.

Protein blotting was performed by a modification of the procedure of Burnette (1981). Crude and purified samples in duplicate were separated on an SDS-PAGE gel as previously described. One half of the gel was stained in Coomassie blue. The other half was used to transfer the protein bands to nitrocellulose paper using a Hoefer TE42 Transphor electrophoresis apparatus or a BioRad transblot cell. The gel and the nitrocellulose paper were assembled into the cassette according to the manufacturer's directions. The tank was filled with blotting transfer buffer (BTB) containing TRIS (25 mM), glycine (192 mM), and methanol (20%, v/v) in distilled water. Samples were run at 200 V for two hours. During this time the current was increased from 0.7 amps to 1.02 amps.

After transfer the sheets were washed for 10 minutes in TRIS buffered saline (TBS) containing TRIS (20 mM) in NaCl (0.5 M), pH 7.5, and then immersed in blocking solution which consisted of BSA (3%) in TBS and agitated gently for 30 minutes. Blots were left overnight at 4°C in blocking solution and shaken again the next morning for 30 minutes at R.T. The sheets were washed for 20 minutes in Tween 20 wash solution (TTBS) containing Tween 20 (0.05%) in TBS, and incubated for 2.5 hours on the shaker with the anti-lectin antiserum diluted 1:500 in antibody buffer (1% BSA in TTBS). The sheets were then washed in two changes, 5 minutes each, of TTBS and incubated for 1 hour in goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (GAR-HRP), diluted 1:3,000 in antibody buffer. After washing twice in TTBS and once in TBS, 5 minutes each wash, the sheets were immersed in HRP color development solution containing 4-chloro-1-napthol prepared just prior to use. This stain (60 µg) was added to 20 ml ice-cold methanol and this added at R.T. to 60 µl ice cold H_2O_2 (30%) in 100 ml TBS. Development of color was allowed to continue until bands were visible (approximately 2-5 minutes). Development was

stopped by immersing the sheets in distilled water with at least one change.

The protein blots were photographed and individual protein bands and standards were stained with Amido black solution consisting of Napthol blue black (0.5 g) dissolved in 225 ml methanol and 50 ml glacial acetic acid, made up to 500 ml with distilled minutes, destained in distilled water for 3-4 hours and rephotographed.

Dot immunoblotting was performed according to Hawkes et al (1982). Purified lectin (1.25 ng/µl) and BSA (30 µg/µl) were dissolved in TBS. 10 µl aliquots of each sample was dotted onto nitrocellulose paper. Duplicate papers were dotted in the same manner and left overnight in blocking solution. The next morning the papers were treated with control serum or antiserum and stained as for the immunoblotting procedure.

10.4 Immunohistochemistry:

10.4.1 Fluorescent Staining.

The jelly coats were removed manually from healthy embryos, which were then further processed in

one of several ways. In some cases, they were oriented in OCT compound and frozen at -20°C. Sections were cut on a histostat (AO 855) at -17°C and mounted on acid-cleaned microscope slides which had been previously coated with rubber cement. Sections were stored at 4°C until use. Prior to staining these sections were fixed in ethanol for 1 minute. Alternatively, whole embryos were fixed prior to sectioning in either 3.7% paraformaldehyde (prepared by adding 3.7 g paraformaldehyde to 70 ml distilled water and heating to 60-70°C, followed by the dropwise addition of 0.1 N NaOH until the solution cleared, and 25 ml of 4 x concentrated SPBS and filtered) or Bouin's fixative (saturated aqueous picric acid, 750 ml; 37-40% formalin, 250 ml; glacial acetic acid, 5 ml). The embryos were embedded in a graded series of polyethylene glycol (25% PEG 400, 50% PEG 400; 75% PEG 400; 100% PEG 400; 1 PEG 400: 1 PEG 1,000 (v/v); PEG 1,000; PEG 1,500, and finally embedded in PEG 1,500). Solutions were changed every hour. Embedded embryos were sectioned.

Sections were stained for one hour with anti-lectin antiserum or pre-immune serum diluted 1:300 in PBS containing BSA (0.5%). After rinsing

in three changes of distilled water (5 minutes each), the slides were treated for 1 hour with FITC-goat anti-rabbit antiserum diluted 1:50 in PBS containing BSA (0.5%). The slides were then rinsed in three changes of distilled water (5 minutes each) and mounted in Dabco (0.25 g in 10 ml 90% glycerin in PBS).

Stained sections were observed with phase and fluorescence microscopy in a Zeiss photomicroscope III and photographed using Kodak Tri-X-Pan or Ektachrome 400 film.

10.4.2 Glucose Oxidase-Antiglucose Oxidase Staining.

For these experiments, the staining kit available from Vector Laboratories was used. The staining involved the use of a secondary anti-rabbit IgG linked to glucose oxidase by an avidin-biotin bridge. Glucose oxidase is detected by the reduction of nitroblue tetrazolium which results in a purple color (Clark, 1982). Sectioned embryos (stage 1½ were washed for 20 minutes in 4 changes of PBS and incubated for 20 minutes in blocking serum (three drops goat serum in 10 ml buffer; 0.01 M $NaKPO_4$ containing 0.145 M NaCl, pH 7.5). After blotting

away the excess serum, the sections were incubated in control serum or anti-lectin antiserum (diluted 1:300 in buffer) and washed in two changes of buffer, 5 minutes for each change. The sections were incubated in biotinylated goat anti-rabbit antiserum (one drop in 10 ml buffer) for 30 minutes and washed in two changes of buffer, 5 minutes for each change. The slides were incubated in ABC-GO reagent, containing avidin and biotinylated glucose oxidase, for 30 minutes and washed again in two changes of buffer. The sections were incubated in glucose oxidase substrate in the dark for 30 minutes and washed for 10 minutes in tap water. The slides were dehydrated in alcohol followed by two changes of xylene and mounted.

RESULTS:

1. LECTIN ACTIVITY IN XENOPUS LAEVIS EMBRYOS

1.1 Extraction of Crude Soluble Lectin Activity:

The first problem addressed in this study was whether or not lectin activity could be detected in early embryos of Xenopus laevis. Although lectins had been reported in early embryos of other vertebrates (Cook et al, 1979), and from other amphibian species (Roberson and Armstrong, 1980), at the time that this project was initiated, no lectins had been reported from early Xenopus embryos. Initially, the procedures used to prepare soluble lectin extracts were modified from Cook et al (1979). However, the lectin extraction solution used by these authors which lacked calcium and contained EDTA was found to inactivate lectin activity from amphibian embryos. Mercaptoethanol, which was also included in the extraction solution of the above authors, had no effect on amphibian lectin activity. In Xenopus embryos a saline solution containing 0.45 mM Ca^{++} and lacking EDTA and mercaptoethanol extracted the maximum amount of stable lectin. The assay used to measure lectin activity in the soluble extracts was essentially the same as the one described by Cook et al (1979), with the exception that

SPBS was the saline used in this study. In spite of the fact that the haemagglutination assay is widely used for the measurement of lectin activity, it is only semiquantitative in nature. In order to make the assay as accurate as possible, the agglutinating activity in each sample was tested in duplicate and the results of the two measurements were averaged. Figure 4 shows an assay of agglutination activity in a crude embryonic extract. The titer of this extract was 256.

Soluble crude lectin extracts from Xenopus laevis embryos at cleavage, blastula, gastrula and neurula stages agglutinate trypsinized rabbit erythrocytes with high titers (Tables 1-4). This lectin activity was stable for one week at 4°C or for several months at -20°C.

1.2 Levels of Lectin Activity During Early Development:

With the knowledge that lectin activity was present in these embryonic extracts, the changes in the level of lectin activity during early development was examined. The results from individual experiments involving the solubilization of lectin from different clutches of frog embryos are shown in Tables 1-4. Table 1 shows the results of eight extractions from cleavage embryos (stage 3-5). A total of 6,310 embryos had a mean activity

FIGURE 4: An assay of haemagglutinating activity. 50 µl aliquots of crude lectin in SPBS was added to 50 µl SPBS in the first well of rows 1 and 2. Increasing dilutions were prepared serially for each subsequent well. 25 µl BSA (1%) and 25 µl rabbit erythrocytes (4%), prepared as described in the text, were added to each well. The plate was read after one hour of incubation at room temperature. Haemagglutination by the lectin is indicated by a fuzzy mat of cells at the bottom of the well. L; lectin-containing wells. Control wells (C) containing no lectin show a distinct dot at the bottom of the well. The titer of the first well is considered to be 2. The titer of this lectin preparation is 256, the reciprocal of the final dilution showing haemagglutination.

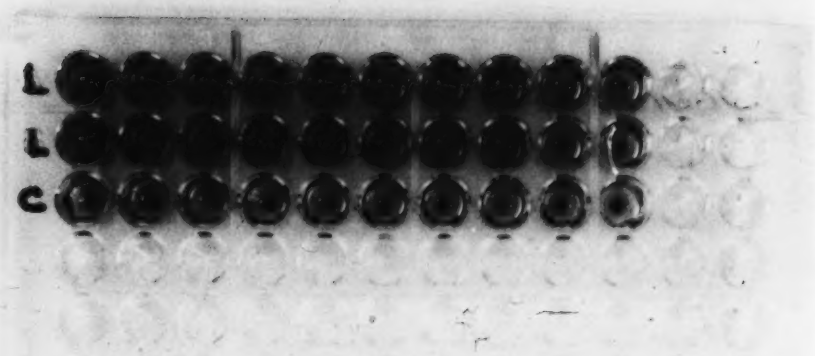

L
L
C

TABLE 1: Soluble lectin activity extracted from cleavage embryos. Eight experiments on different clutches of embryos are shown here. A total of 6,310 cleavage embryos (stage 3-5) were used. Activity (HU/embryo), specific activity (HU/µg protein) and protein/embryo were transformed to Log_{10} values, and the mean ± standard error error is given for these values. Activity (HU) and protein were measured as described in the text.

EXP	NO. OF EMBRYOS	ACTIVITY (TOTAL HU)	HU/EMBRYO	$LOG_{10}\frac{HU}{EMB}$	TOTAL SOLUBLE PROTEIN (μg)	SOLUBLE PROT/EMB	$LOG_{10}\frac{PROT}{EMB}$	SPECIFIC ACTIVITY $\frac{HU}{\mu g}$	$LOG_{10}\frac{HU}{\mu g}$
1.	300	5414400	18048	4.256	7200	24.0	1.380	752	2.876
2.	160	25779	161	2.207	4864	30.4	1.483	5	0.724
3.	460	8654256	18814	4.274	10764	23.4	1.369	804	2.905
4.	500	37335000	74670	4.873	14250	28.5	1.455	2620	3.418
5.	3761	118066620	31392	4.497	36108	9.6	0.982	3270	3.515
6.	765	105332400	137689	5.139	6502	8.5	0.929	16200	4.210
7.	289	982736	3400	3.531	3613	12.5	1.100	272	2.435
8.	75	491749	6557	3.817	617	8.2	0.915	797	2.901
MEAN			36,341	4.07 ± 0.32		18.14	1.20 ± 0.08	3090	2.87 ± 0.36

TABLE 2: Soluble lectin activity extracted from blastula embryos. Thirteen experiments each performed on a different clutch of embryos are shown here. A total of 5,816 blastula embryos (stage 7-9) were used. Log_{10} mean values $\pm$ standard error are shown for HU/embryo, HU/μg protein and protein/embryo, as in Table 1.

EXP	NO. OF EMBRYOS	ACTIVITY (TOTAL HU)	HU/EMBRYO	$\log_{10}\frac{HU}{EMB}$	TOTAL SOLUBLE PROTEIN (μg)	SOLUBLE PROT/EMB	$\log_{10}\frac{PROT}{EMB}$	SPECIFIC ACTIVITY $\frac{HU}{\mu g}$	$\log_{10}\frac{HU}{\mu g}$
1.	300	24570000	81900	4.913	9000	30.0	1.477	2730	3.436
2.	1000	16500000	16500	4.217	25000	25.0	1.398	660	2.820
3.	1813	14919810	8229	3.915	23570	13.0	1.114	633	2.801
4.	112	58861100	525546	5.721	5186	46.3	1.666	11350	4.055
5.	330	57624000	174618	5.242	10296	31.2	1.494	5597	3.748
6.	359	713592	1988	3.298	4488	12.5	1.097	159	2.200
7.	293	6489000	22147	4.345	12600	43.0	1.633	515	2.712
8.	423	5639660	13333	4.125	2834	6.7	0.826	1990	3.299
9.	474	34241200	72239	4.859	24458	51.6	1.713	1400	3.146
10.	250	1048125	4193	3.622	5375	21.5	1.332	195	2.290
11.	45	697680	15504	4.190	612	13.6	1.134	1140	3.057
12.	210	3611790	17199	4.236	3087	14.7	1.167	1170	3.068
13.	207	160200	774	2.889	1780	8.6	0.934	90	1.954
MEAN			73,398	4.27 ± 0.21		24.4	1.31 ± 0.08	2,125	2.97 ± 0.17

TABLE 3: Soluble lectin activity extracted from gastrula embryos. Fifteen experiments each performed on a different clutch of embryos are shown. A total of 8,715 gastrula embryos (stage 10, 11) were used. Log_{10} mean values $\pm$ standard error are given for HU/embryo, HU/µg protein and protein/embryo, as in Table 1.

EXP	NO. OF EMBRYOS	ACTIVITY (TOTAL HU)	HU/EMBRYO	$LOG_{10}\frac{HU}{EMB}$	TOTAL SOLUBLE PROTEIN (μg)	SOLUBLE PROT/EMB	$LOG_{10}\frac{PROT}{EMB}$	SPECIFIC ACTIVITY $\frac{HU}{\mu g}$	$LOG_{10}\frac{HU}{\mu g}$
1.	700	105806400	151152	5.179	19740	28.2	1.450	5360	3.729
2.	960	32747520	34112	4.533	19968	20.8	1.318	1640	3.215
3.	256	4828160	18860	4.276	5888	23.0	1.362	820	2.914
4.	272	2193450	8064	3.907	10455	38.4	1.585	210	2.322
5.	664	45910800	69143	4.840	13080	19.7	1.294	3510	3.545
6.	916	5242268	5723	3.758	27022	29.5	1.470	194	2.288
7.	171	125024000	731138	5.864	3214	18.8	1.274	38900	4.590
8.	516	640825800	1241911	6.094	8153	15.8	1.199	78600	4.895
9.	997	159012000	162755	5.212	15144	15.5	1.190	10500	4.021
10.	213	10224000	48000	4.681	5112	24.0	1.380	2000	3.300
11.	95	26647500	280500	5.448	1425	15.0	1.176	18700	4.272
12.	265	53424000	201600	5.305	5936	22.4	1.350	9000	3.954
13.	450	561600	1248	3.096	4320	9.6	0.982	130	2.114
14.	894	81772860	91469	4.961	33241	37.2	1.570	2460	3.391
15.	1366	61567000	45071	4.654	19670	14.4	1.158	3130	3.500
MEAN			206,050	4.79±0.21		22.15	1.32±0.04	11,677	3.47±0.21

TABLE 4: Soluble lectin activity extracted from neurula embryos. Eight experiments each from a different clutch of embryos are shown. A total of 4,213 embryos at the neurula stage (20-22) were used. Log_{10} mean values $\pm$ standard error are given for HU/embryo, HU/µg protein and protein/embryo, as in Table 1.

EXP	NO. OF EMBRYOS	ACTIVITY (TOTAL HU)	HU/EMBRYO	$LOG_{10}\frac{HU}{EMB}$	TOTAL SOLUBLE PROTEIN (µg)	SOLUBLE PROT/EMB	$LOG_{10}\frac{PROT}{EMB}$	SPECIFIC ACTIVITY $\frac{HU}{\mu g}$	$LOG_{10}\frac{HU}{\mu g}$
1.	370	14188200	38346	4.584	13912	37.6	1.575	1020	3.009
2.	320	18880	59	1.771	9440	29.5	1.470	2	0.301
3.	410	32742600	79860	4.902	14883	36.3	1.560	2200	3.342
4.	400	4508000	11270	4.052	12880	32.2	1.508	350	2.544
5.	150	568000	3787	3.578	7200	48.0	1.681	79	1.897
6.	909	10908	12	1.079	5454	6.0	0.778	2	0.301
7.	1515	109195240	72076	4.858	18634	12.3	1.090	5860	3.768
8.	139	364080	2619	3.418	2960	21.3	1.328	123	2.090
MEAN	527		26,004	3.53 ± 0.5		27.9	1.37 ± 0.11	1205	2.16 ± 0.46

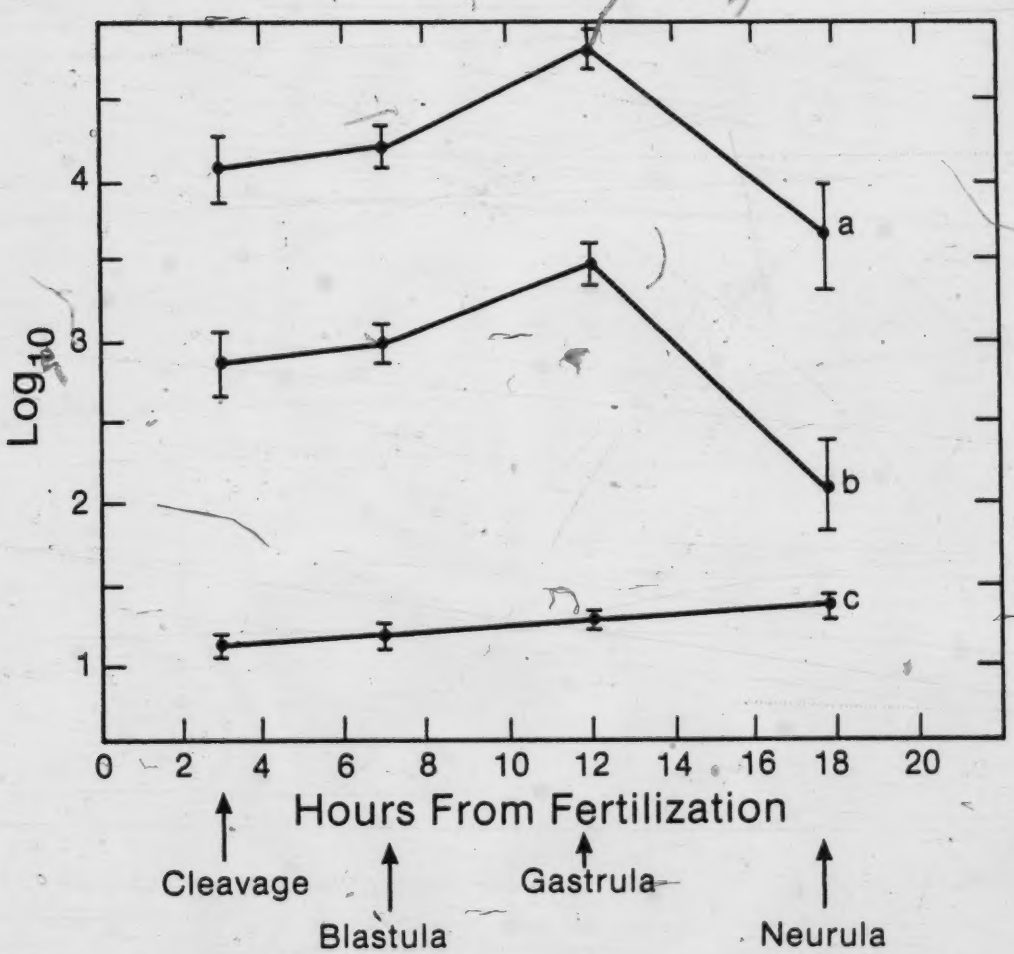
of 36,341 HU per embryo and a specific activity of 3,090 HU per µg protein. An average of 18.14 µg of soluble protein was extracted per embryo. Table 2 shows the values obtained from 13 extractions of blastula embryos (stages 7-9). The 5,816 embryos used showed a mean activity of 73,398 HU per embryo and a specific activity of 2,125 HU per µg protein. An average of 24.4 µg of soluble protein was extracted under these conditions. Table 3 shows the values obtained from 15 extractions of gastrula embryos (stages 10 and 11). A total of 8,715 embryos had a mean activity of 206,050 HU per embryo and a specific activity of 11,677 HU per µg protein. The average amount of soluble protein extracted under these conditions was 22.15 µg per embryo. Finally, Table 4 shows the results of 8 extractions carried out on a total of 4,213 neurula embryos (stages 20-22). These embryos had a mean activity of 26,004 HU per embryo, a specific activity of 1,205 HU per µg protein and an average yield of 27.9 µg of soluble protein extracted per embryo.

Of approximately 120 lectin extractions involving 48,000 embryos undertaken during the course of this study, the experiments shown in Tables 1-4 were selected because they were conducted under the same conditions, the embryonic stages fell exactly within the ranges indicated and

they all involved a single clutch of embryos. Even with these precautions, however, there is considerable variation in lectin activity and in the amount of protein extracted within a single stage. When the data are converted to Log_{10} values and plotted against time of development after fertilization, the embryos show a stage-dependent change in the level of soluble protein extracted, (Figure 5c), lectin activity per embryo (Figure 5a) and lectin activity per µg of protein (Figure 5b). Gastrula embryos showed the highest level of activity and neurula embryo had the lowest level. This difference was found to be significant ($0.01<p<0.025$ for gastrula embryos versus all others and $0.01<p<0.025$ for gastrula versus neurula embryos). There was no significant difference between blastula, cleavage or neurula embryos with respect to activity. Lectin activity (HU per µg protein or HU per embryo) was found to increase linearly from 2 to 12 hours ($p<0.05$) and to decrease significantly from 12 to 18 hours ($p<0.01$ for regression against hours).

In contrast to lectin activity, the level of protein solubilized under these conditions increased gradually from the earliest stage studied (cleavage) to the latest (neurula).

FIGURE 5: Change in levels of lectin activity/embryo (a), specific activity (lectin activity/μg protein) (b), and μg soluble protein extracted/embryo (c) at successive developmental stages. Numerical data were transformed to Log_{10} values. Each point represents the mean $Log_{10} \pm$ standard error at one of four stages: cleavage (8 experiments); blastula (13 experiments); gastrula (15 experiments); and neurula (8 experiments). Each experiment was conducted on a different clutch of embryos. The number of embryos in a single experiment ranged from 45 to 3,761.


Log10
4
3
2
1
a
b
c
0
2
4
6
8
10
12
14
16
18
20
Hours From Fertilization
Cleavage
Blastula
Gastrula
Neurula

1.3 Lectin Activity in Xenopus Ovarian Tissue:

Lectin extracts prepared from minced ovarian tissue also contained a high level of lectin activity. The supernatant obtained from one extraction contained 0.077 µg/µl of soluble protein and 8×10^6 HU in 50 µl. The specific activity of this extract was 2×10^9 HU per mg protein. A direct comparison to the embryonic extracts is not possible since only one homogenate of this tissue was prepared and because the ratio of tissue mass to the volume of extraction solution used was not the same in both procedures.

1.4 Lectin Activity in the Blastocoele Cavity:

A small volume (0.1 µl) of fluid was withdrawn from the blastocoele cavities of two Xenopus embryos (stage 8) with a Hamilton syringe. This volume was diluted in 75 µl of SPBS and 50 µl was used in the haemagglutination assay. The titer of this solution was 2048, or 3.07×10^4 HU per µl of blastocoele fluid. Since the volume of the blastocoele fluid contents is unknown, it is not possible to calculate the total HU present there. In this experiment, however, 1.54×10^3 HU was removed from each embryo, compared to the average value of 73.4×10^3 HU removed from entire blastula embryos (Table 2).

2. ANALYSIS OF CRUDE LECTIN SUPERNATANTS

2.1 Lectin Specificity:

The presence of lectin activity was assessed by the ability of soluble extracts to agglutinate erythrocytes. The Xenopus embryonic lectin was further defined by determining its carbohydrate specificity. Generally, lectin molecules show a capacity to bind to specific monosaccharides. In addition, they may show an increased preference for a specific sugar if it is present in a particular configuration in a disaccharide or a polysaccharide. The specificity of a lectin is usually measured by the ability of simple sugars in solution to inhibit the agglutination reaction. However, the ability of a soluble saccharide to inhibit the agglutination reaction may change when that sugar is attached to an insoluble matrix. The three-dimensional presentation of the carbohydrate molecule appears to be an important factor influencing the degree of binding by a particular lectin (Lee et al, 1984). Therefore, two approaches were undertaken to define the specificity of the Xenopus lectin; firstly, a measure of the inhibition of agglutination by simple monosaccharides and disaccharides and secondly, the ability of sugars bound to an insoluble material (immunoadsorbents) to

remove the agglutinating activity of crude lectin extracts.

2.1.1 Inhibition by Simple Sugars.

The lectin activity in crude supernatants of Xenopus laevis embryos could be inhibited by galactose and saccharides containing α- or β-D-galactose residues. The concentration of simple saccharides (mM) required to inhibit 4 HU of lectin activity is shown in Table 5. The disaccharide thiodigalactoside having two β-galactose residues is the most effective inhibitor. Lactose, which has one β-galactose residue is the second most effective, followed by melibiose which has one α-galactose residue. Of the monosaccharides tested, methylα-D-galactopyranoside was a more effective inhibitor than its β-anomer. N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, methyl-α-D-mannopyranoside and L-fucose did not change the lectin activity when tested at concentrations of 25 mM, indicating that the lectin has little affinity for these sugars. The saccharides tested inhibited extracts of the four different stages to a similar degree. The results shown here are similar to those reported by Roberson and Barondes (1982) who tested the ability of the

TABLE 5: Inhibition of lectin activity in extracts of embryos from cleavage to neurula stage. Values represent concentration (mM) of substance required for complete inhibition of 4 HU. The figures in parenthesis represent the number of experiments showing these values.

INHIBITOR	CLEAVAGE	BLASTULA	GASTRULA	NEURULA
LACTOSE β-D-GAL(1-4)GLC	6.25(1)	1.56(1) 6.25(2)	6.25(1) 3.13(1) 12.5 (1)	3.13(1) 6.25(1)
THIODIGALACTOSIDE β-D-GAL(1-1)β-D-THIOGAL	1.56(1)	1.56(2) 0.39(1)	1.56(1) 3.13(2)	3.13(1) 0.78(1)
MELIBIOSE α-D-GAL(1-6)GLC		12.5 (2)	25	12.5 (1)
METHYL α-D-GALACTOPYRANOSIDE		6.25(1) 12.5 (1)		
METHYL β-D-GALACTOPYRANOSIDE		25 (1) 50 (1)		
FUCOSE		50 (2)	100 (1)	100 (1)
METHYL α-D-MANNOPYRANOSIDE	25(1)	25 (3)	25 (1)	25 (1)
N-ACETYL-D-GALACTOSAMINE	25(1)	25 (3)	25 (3)	25 (2)
N-ACETYL-D-GLUCOSAMINE		50 (3)	100 (1)	100 (1)

same sugars to inhibit the activity of Xenopus oocyte lectin. The values shown here are not directly comparable to those of the above authors, however, since they reported the concentration of sugars necessary to inhibit 50% of the lectin activity. The chick embryonic lectins show a similar pattern of inhibition by galactose, although in this case β-galactose residues appear to be more effective than α-galactose residues (Zalik et al, 1983 and Cook et al, 1979).

2.1.2 Inhibition by Bound Sugars.

The bound sugars used in these studies were immunoadsorbents which were kindly donated by Dr. R. Lemieux of the Chemistry Department, University of Alberta and obtained from ChemBioMed, U. of Alberta. These substances bear synthetically derived sugars of known structure attached to an insoluble silica matrix. Table 6 shows the ability of these immunoadsorbents to remove the haemagglutinating activity from lectin extracts, expressed as percent inhibition. In this table -R represents the linking arm to the solid matrix. Results from blastula and gastrula stages are shown. At both stages, the disaccharide DGal(α1-3)DGalβ1-R is the best inhibitor, since it removes most of the lectin

TABLE 6: The effect of different saccharide-linked immunoadsorbents on the agglutinating activity of crude lectin extracts. For each test, soluble extracts were incubated with IA (54,000 HU/mg IA) for 2 hours at 4°C with constant shaking. The % inhibition represents the decrease in activity of the supernatant after incubation with the IA compared to the activity of the original extract. -R represents the linking arm to the solid matrix. Each value is the mean of three separate measurements using extracts from different clutches of embryos.

Saccharide	% INHIBITION	
	Blastula	Gastrula
None	0	0
DGlcα1-R	0	0
DGalβ1-R	66	61
DGalα1-R	65	76
Lactose (DGal(β1-- 4)DGlcβ1-- R)	44	50
DGalNAcα1-R	28	11
DGal(α1-- 3)DGalβ1-R	88	94
DGal(α1-- 4)DGalβ1-R	79	88
DGal(β1-- 3)DGalNAcα1-R	84	92
DGal(β1-- 3)DGlcNAcβ1-R	50	75
DGal(β1-- 4)DGlcNAcβ1-R	67	57

activity from the extract (88-94%). Strong inhibition was also shown by DGal(β1-3)DGalNAcα1-R (84-92%) and DGal(α1-4)DGalβ1-R (79-88%). Weaker inhibition was obtained with DGal(β1-3)DGlcNAcβ1-R (50-75%), DGal(β1-4)DGlcNAcβ1-R (57-67%), lactose (44-50%) and the monosaccharides DGalβ1-R (61-66%) and DGalα1-R (65-76%).

2.2 The Requirement for Calcium:

The divalent cation calcium is necessary to maximize and stabilize the lectin activity. The inclusion of calcium in the buffer used during the haemagglutination assay increased the lectin activity 16-fold compared to lectin diluted in Ca^{++} Mg^{++}-free buffer. In addition, the chelating agents EDTA and EGTA were found to be strong inhibitors of agglutination. In two assays involving crude blastula extract, EDTA or EGTA at a concentration of 0.125 to 0.25 mM, completely inhibited 4 HU lectin activity. Not surprisingly, when lectin extracts were dialysed against Ca^{++} Mg^{++}-free SPBS containing EDTA or EGTA, lectin activity was completely abolished (Table 7). When these inactivated lectin extracts were dialysed further against SPBS containing Ca^{++} and Mg^{++} the inhibition was reversed. As shown in Table 7, the activity of

TABLE 7: The reversible effect of chelating agents on the activity of soluble lectin extracts. The activity of six supernatants was measured before treatment (A), after dialysis against five changes of CMF-SPBS containing EDTA (0.002 M) or EGTA (0.002 M) (B), and again after dialysis against five changes of complete SPBS (C). Supernatants 2 and 5 were crude soluble extracts and supernatants 1,3,4 and 6 were purified extracts, all from gastrula embryos.

	A INITIAL TITER	B TITER AFTER DIALYSIS AGAINST CMF-SPBS WITH CHELATING AGENT	C TITER AFTER DIALYSIS AGAINST SPBS
		EDTA	
1.	2048	0	1024
2.	4096	0	2048
3.	8	0	8
		EGTA	
4.	1024	0	1024
5.	2048	0	2048
6.	8	0	8

EDTA-treated extracts returned to 50% or 100% of the original value and extracts exposed to EGTA completely regained their activity.

2.3 The Effects of Temperature and Trypsin Treatment:

Table 8 shows that the lectin activity is sensitive to heat treatment. Heating at 100°C for 5 minutes completely abolished the activity of a crude gastrula supernatant containing 48 HU in 50 µl. The titer was unaffected by heating at 37°C for 60 minutes and showed an increase of 50% when heated at 60°C for 50 minutes. This increase may represent a slight activation of the lectin but more likely it is due to the semiquantitative nature of the assay. A 50% change in activity is brought about by a difference of a single dilution and the repeat titrations undertaken for these tests quite frequently differed by one dilution. In spite of the care taken in setting up the assay minor inequities in pipetting or erythrocyte activity could produce quantitative variations of this nature.

The activity of the Xenopus lectin is also trypsin sensitive. When treated with trypsin at a concentration of 1 mg/ml at 37°C for 60 minutes the activity of 50 µl of gastrula lectin was reduced from 4,096 HU to 24 HU. This represents a 99.4% decrease in activity.

TABLE 8: The effect of heat treatment and trypsin treatment on lectin activity. The activity in 50 µl crude gastrula extract was recorded at room temperature (1,3), after heating (2,4 and 5) and after incubation with an equal volume of SPBS containing trypsin, Sigma, type III, at a concentration of 1 mg/ml, at 37°C for 60 minutes, (6).

	TREATMENT	INITIAL TITER (HU/50 µl)	FINAL TITER HU/50 µl)
1.	R.T. 50 min.	64	64
2.	60°C 50 min.	64	96
3.	R.T. 5 min.	48	48
4.	100°C 5 min.	48	0
5.	37°C 60 min.	4096	4096
6.	37°C 60 min. Trypsin, 1mg/ml	4096	24

3. LECTIN PURIFICATION

Approximately 25 proteins can be resolved by SDS-PAGE of crude soluble extracts of Xenopus laevis embryos (Figure 13). Several different approaches were undertaken to isolate pure lectin fractions from this heterogeneous mixture. All of the methods took advantage of the specificity of the lectin molecule for α- or β-D-galactose.

3.1 Batch Adsorption to αGal-Immunoadsorbent:

α-Gal IA, which removed 65-76% of the lectin activity from crude supernatants (Table 6) was available in sufficient quantities to be used for affinity purification. The IA was added to lectin supernatants in a ratio of 10 mg IA to 1 ml of crude extract having an average titer of 261,590 HU/50 µl. Bound lectin was eluted from the IA by washing with NH_4OH (1%) in NaCl (0.07%) with or without lactose (0.3 M), as described in Materials and Methods. The results of five purifications using a total of 1,114 blastula embryos are shown in Table 9;a. The crude extracts had a mean activity of 1,394,976 HU. Of this, 94,463 HU (6.8%) remained unbound in solution and 1,063,354 HU (76.2%) was recovered after elution of the adsorbed material. The increase in specific activity of the purified material over that of the crude extract (the relative specific activity) was 19-fold. Table 9;b shows

TABLE 9: Batch adsorption of galactose-specific lectin on immunoadsorbents possessing α-DGal residues (0.3-0.5 moles/gm). Bound material was eluted with NH_4OH (1%) in NaCl (0.07%) with or without 0.3 M lactose. Values shown for activity (HU) and specific activity of the sample (HU/mg protein) represent the means of five separate purification ± standard error of the mean. a. Extracts from blastula embryos. At this stage, purified lectin represented 7.2% of crude soluble protein. b. Extracts from gastrula embryos. The purified lectin represented 8.8% of crude soluble protein. The relative specific activity in the activity of the fraction over that of the original crude extract.

Stage of Purification	Activity (HU)	Specific Activity of Sample (HU/mg protein)	% of Original Activity	Relative Specific Activity
Crude Embryo Extract	a. 1 394 976 ± 490 000	957 000 ± 303 000	100	1
	b. 4 837 920 ± 1 200 000	1 166 000 ± 572 000	100	1
Unbound Activity Remaining in Solution after Absorption	a. 94 463	107 000	6.8	0.11
	b. 807.520	268 000	16.7	0.25
Purified Lectin Recovered by Elution of Bound Material	a. 1 063 354 ± 127 000	18 500 000 ± 5 900 000	76.2	19
	b. 3 371 936 ± 1 860 000	50 220 000 ± 21 300 000	69.7	43

the results obtained from five purifications using a total of 2,565 gastrula embryos. The crude extract had an initial activity of 4,837,920 HU. Of this, 807,520 HU (16.7%) remained unbound and 3,371,936 HU (69.7%) was recovered after elution. The increase in the relative specific activity was 43-fold.

3.2 Affinity Chromatography:

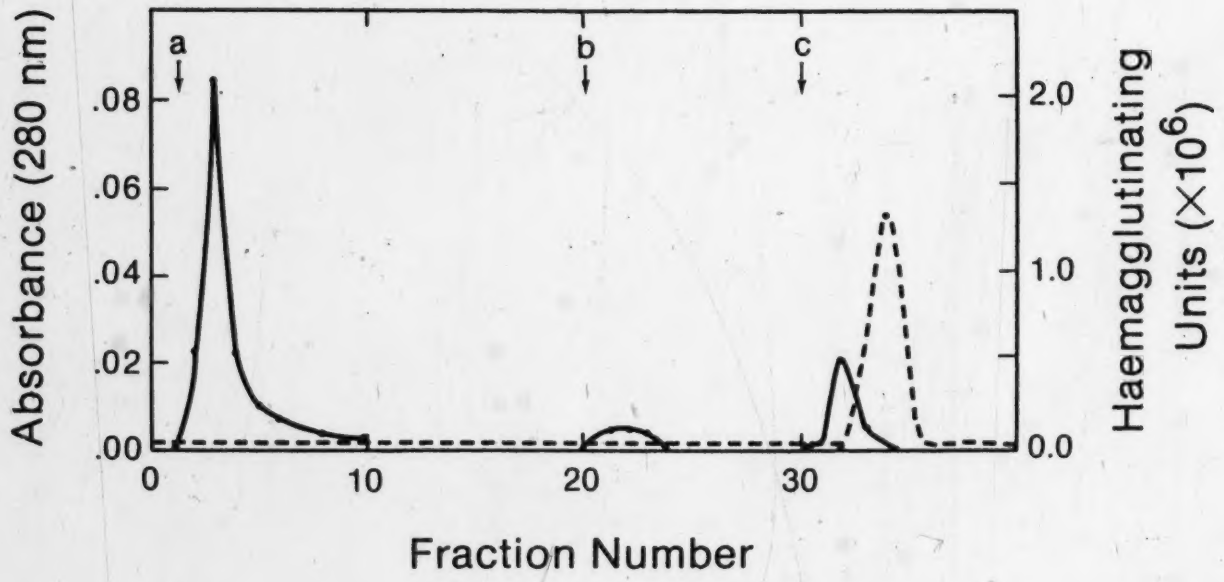
3.2.1 ρ-Aminophenyl-β-D-lactoside, Sepharose 4B Column.

The Xenopus galactose-binding lectin could also be successfully purified on an affinity column containing APL-Sepharose 4B. One such purification involving 164 gastrula embryos is shown in Figure 6. The activity of the original crude extract was 1,721,440 HU. Of this, 55,624 HU (3.2%) passed through the column without binding. 1,656,720 HU (96.2%) were recovered after elution with SPBS + 0.3M lactose, representing a 34-fold increase in the relative specific activity.

3.2.2 Melibiose-Selectin 4 Column.

Several attempts were made to purify the lectin by affinity chromatography using a column contain-

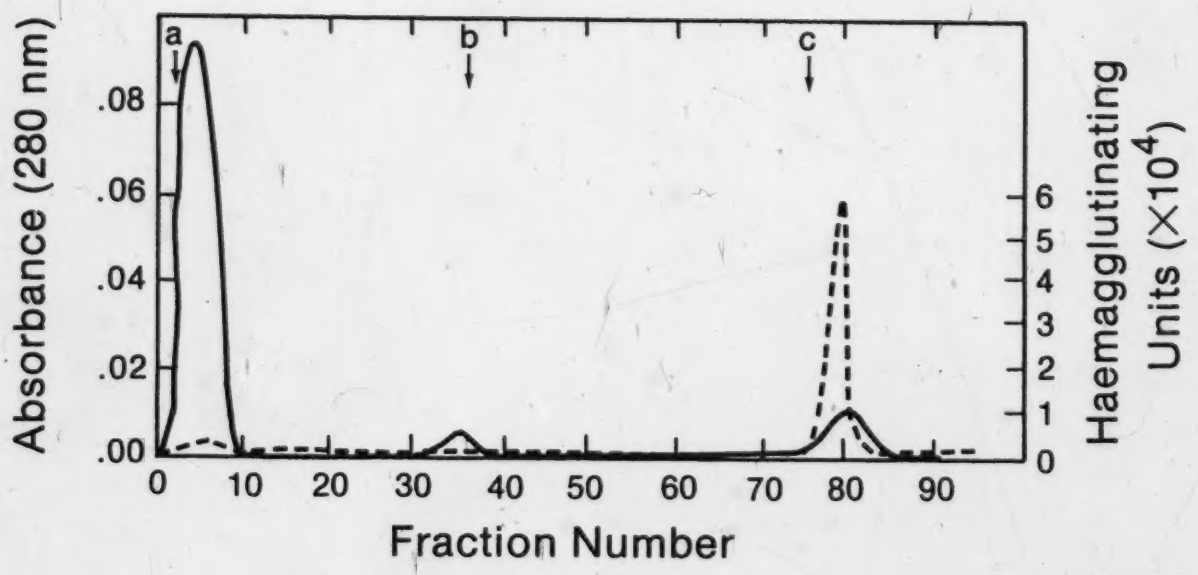
FIGURE 6: Affinity chromatography of crude gastrula lectin on a column (0.5 x 20 cm) of ρ-aminophenylβ-D-lactoside. 1,721,440 HU was added to the column which was washed with 70 ml SPBS (a), 35 ml SPBS + 0.3 M sucrose (b) and SPBS + 0.3 M lactose (c). Fractions were separately dialysed against SPBS and the total activity in each fraction was measured (------). 96.2% of the original activity was recovered in this experiment (1,656,720 HU).


a
b
c
.08
.06
.04
.02
.00
0
10
20
30
2.0
1.0
0.0
Absorbance (280 nm)
Haemagglutinating Units ($\times 10^6$)
Fraction Number

ing Selectin 4. This matrix possesses bound melibiose residues and was successfully used by Roberson and Barondes (1982) to purify the galactose-binding lectin from Xenopus oocytes. The results of one experiment with extract from 65 blastula embryos is shown in Figure 7; 2 ml of extract containing 5,184,000 HU was added to the column. Of this, 2,300 HU was detected in the various wash steps and 76,261 HU (1.47%) was eluted by washing with SPBS containing 0.3 M lactose. The remaining 98.6% of the activity could not be recovered. A second attempt using this procedure and another extract from blastula embryos gave similar results.

The method of choice for the purification of the lectin throughout this project was by batch adsorption to α-Gal IA. This method gave good recoveries of lectin activity and was easily and quickly carried out. Experiments conducted with lectin purified on columns of either APL-Sepharose or Selectin 4 will be noted in the text. This investigation involved the purification of crude extracts from a total of 35,000 embryos from 65 separate clutches. Each purification was conducted with embryos from a single developmental stage.

FIGURE 7: Affinity chromatography of crude blastula extract on a column (0.5 x 20 cm) of Selectin-4. 5,184,000 HU was added to the column which was washed with 100 ml SPBS (a), 20 ml SPBS + 0.3 M sucrose (b), and 20 ml SPBS + 0.3 M lactose (c). All fractions after number 30 were dialysed separately against SPBS and the total activity in each fraction was measured (-------). 14.7% of the original activity was recovered in this experiment (76,261 HU).


a
b
c
.08
.06
.04
.02
.00
0
10
20
30
40
50
60
70
80
90
Absorbance (280 nm)
Fraction Number
6
5
4
3
2
1
0
Haemagglutinating Units (×10⁴)

3.3 Chloroform-Methanol Treatment:

The amphibian embryo contains considerable amounts of yolk. The yolk platelets in Xenopus are composed largely of the phosphoprotein phosvitin, the lipophosphoprotein lipovitellin and bound lipid (Wallace, 1963). Much of this yolk material is liberated upon extraction of the soluble substances of these embryos. The lipid from yolk granules and other cellular lipids result in a crude extract with a very turbid appearance. Following purification by any of the methods described above, the lectin solution appears clear. However, some of these lipoproteins may be closely associated with the lectin molecule. Alternatively, some lipid might bind non-specifically to the matrices used for purification. In either case, the contaminating material could be expected to appear in the purified supernatants along with the lectin activity. That this was indeed the case was suspected when polyacrylamide gel electrophoresis of purified lectin gave variable results involving a number of bands (described in Section 5.1). Therefore, a final purification step involving the treatment of lectin supernatants with a chloroform-methanol mixture (2:1, v/v) was introduced. The results of this treatment are shown in Table 10. The first experiment (a) involved the treatment of crude gastrula extract and resulted in the recovery of 60% of

TABLE 10: Treatment of crude and purified lectin samples, all from gastrula embryos, with chloroform-methanol (2:1 v/v). Equal volumes of chloroform-methanol solution were shaken with the lectin extracts for 15 minutes at room temperature and the mixture was centrifuged at 900 x g for 10 minutes in a clinical centrifuge. The upper aqueous layer was removed and tested for haemagglutinating activity.

EXTRACT	#HU		% RECOVERY
	BEFORE TREATMENT	AFTER TREATMENT	
a. CRUDE	5.9×10^6	3.5×10^6	60
b. CRUDE	0.98×10^6	1.97×10^6	200
c. PURE	0.12×10^6	0.06×10^6	50

the activity. A second experiment (b) involved crude gastrula extract and resulted in a 2-fold increase in the activity after treatment. In the third trial (c), purified lectin from gastrula embryos was treated and 50% of the activity was recovered. The results of these experiments vary from one another and the final HU are not the same as the activity of the original extract. However, because of the semiquantitative nature of the agglutination assay which involves serial 2-fold dilutions of the lectin extract, these values indicate that essentially all of the activity was recovered after treatment. The treatment was equally successful using either crude or purified extracts but for convenience most of the treatments were carried out on crude material. Chloroform-methanol treated extracts used for experiments in this study will be noted in the text.

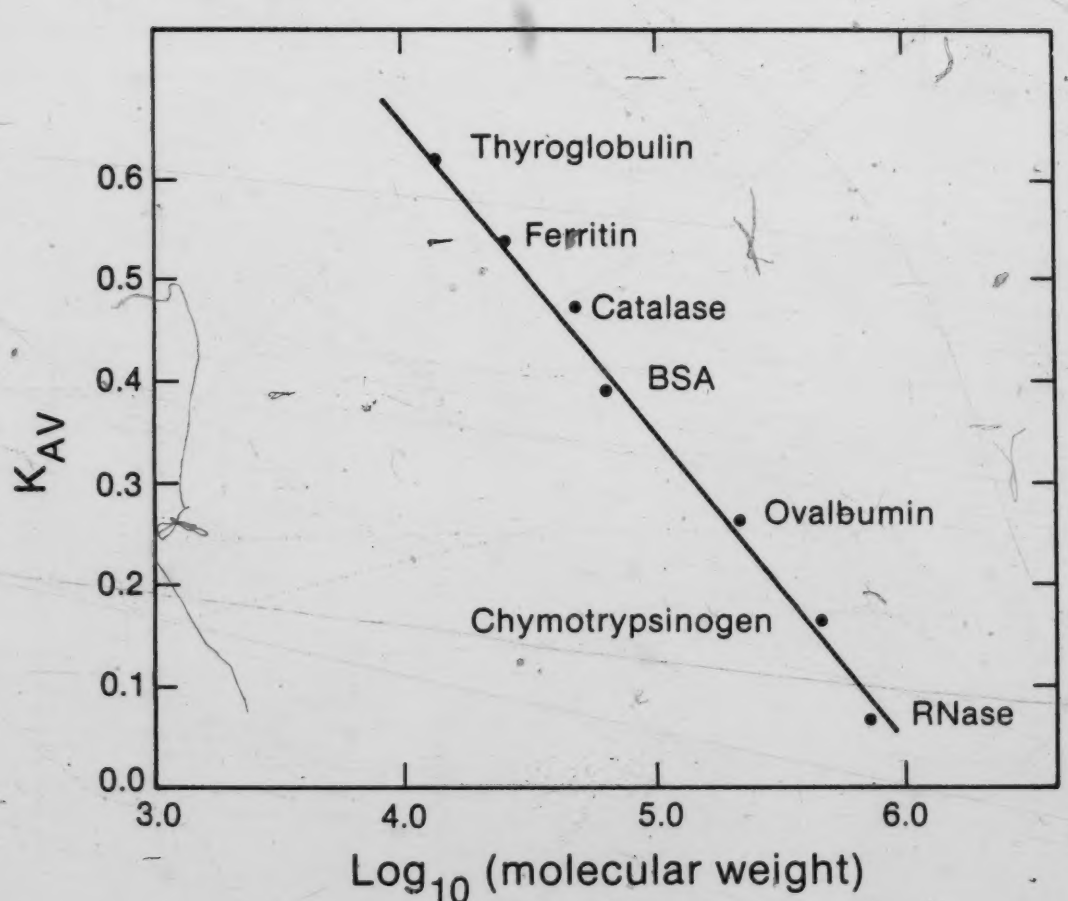
4. GEL FILTRATION CHROMATOGRAPHY

Gel filtration chromatography was carried out in order to determine the nature of the lectin under non-denaturing conditions. In particular, it is important to know the molecular size of the lectin and its capacity to self-associate, since these properties may reflect the physiological condition of the lectin. Sephacryl (Pharmacia Fine Chemicals) was chosen as a gel filtration

medium because it is composed of dextran cross-linked with bisacrylamide and therefore, unlike Sepharose, contains no galactose units to which the lectin may bind. Initial experiments were performed using a column containing Sephacryl S200 which has a fractionation range of 5,000 to 250,000 molecular weight (Pharmacia). When crude or purified lectin activity from all stages was introduced to the column and eluted with SPBS it appeared almost exclusively in the void volume. Several attempts were made to reduce the aggregate size of the lectin molecule, including elution in the presence of the detergent, octyl glucoside (0.25%), and also elution with SPBS containing NaCl (1 M). In all cases the results were similar to those obtained by elution with SPBS alone.

Later experiments were carried out using a column of Sephacryl S-300 SF which has a fractionation range of 10,000 to 1.5 x 10^6 molecular weight (Pharmacia). The dimensions of the column were 2.6 x 40 cm. Standard curves for the determination of molecular weight were prepared for each elution buffer used. A typical standard curve is shown in Figure 8. In this case the elution buffer was SPBS with lactose (0.1 M). In all cases the volume of the fractions collected was 3.5 ml.

FIGURE 8: Molecular weight standards for gel filtration on Sephacryl S-300 SF. The column (2.6 x 40 cm) was equilibrated with SPBS + lactose (0.1 M). Fractions of 3.5 ml were collected. $K_{av}=(v_e-v_o)/(v_t-v_o)$ where v_e=elution volume, v_o=void volume, and v_t=total bed volume. The molecular weight standards were: thyroglobulin, 669,000; ferritin, 440,000; catalase, 232,000; BSA, 67,000; ovalbumin, 45,000; chymotrypsinogen, 25,000 and ribonuclease, 13,700.


Thyroglobulin
Ferritin
Catalase
BSA
Ovalbumin
Chymotrypsinogen
RNase
K_{AV}
0.0
0.1
0.2
0.3
0.4
0.5
0.6
3.0
4.0
5.0
6.0
Log_{10} (molecular weight)

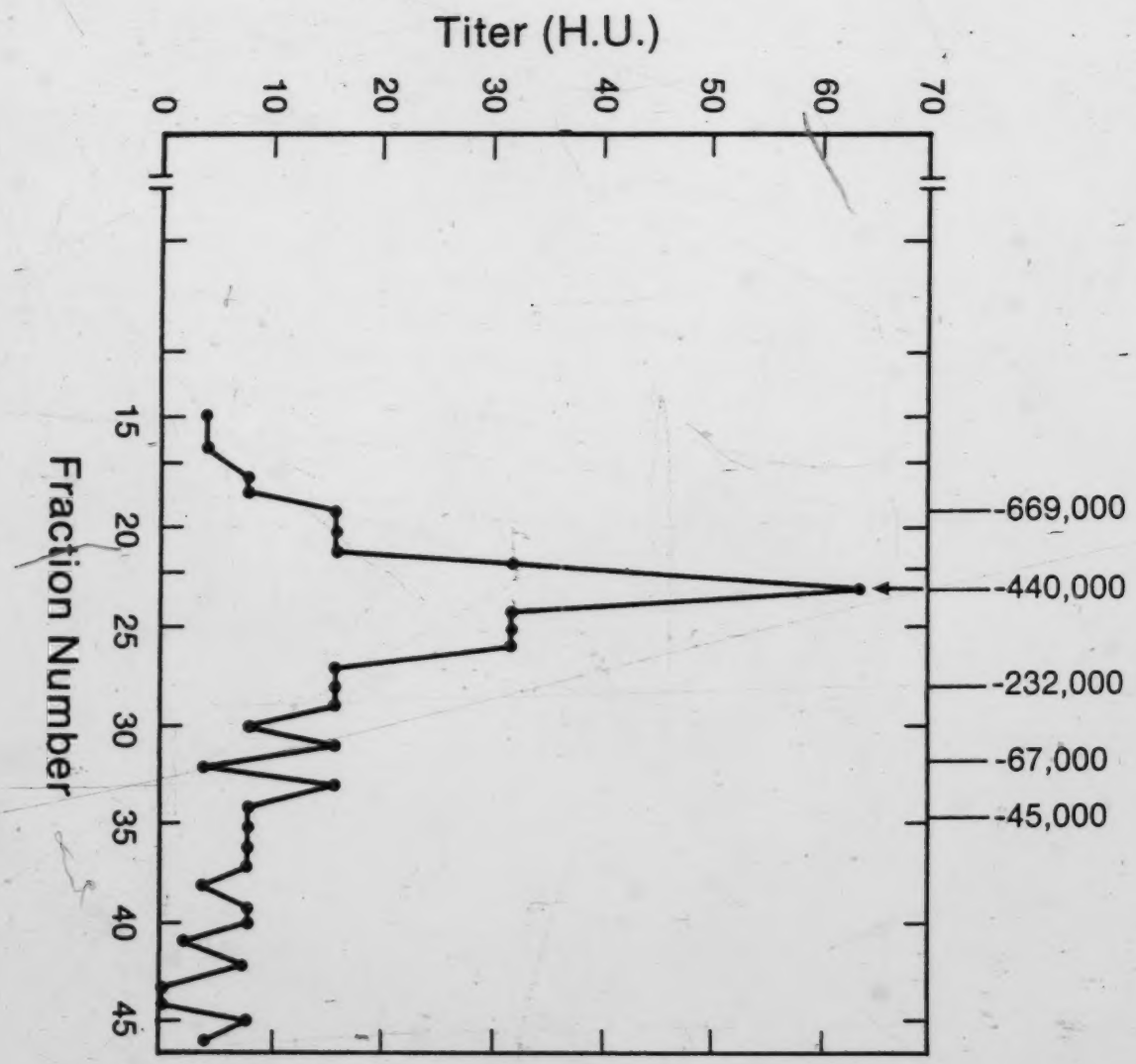
4.1 Elution with SPBS:

A typical experiment showing lectin activity eluted from the gel filtration column with SPBS is shown in Figure 9. Purified lectin from cleavage embryos (0.23 mg) was added to the column. Only 6.3% of the original activity was recovered. The apparent molecular weight of the peak fraction in Figure 9 is 440,000. There is evidence, however, that the protein is being retarded by the matrix of the column. Firstly, because of the low recovery it is possible that much of the protein interacted non-specifically with the column material and was not eluted with SPBS. Secondly, some activity continues to appear in fractions well beyond the volume necessary to elute the smallest standard protein. Other experiments using crude or purified extracts showed results similar to these when chromatographed under the same conditions. The apparent molecular weight of the peak fraction shown in Figure 9 was not consistently obtained. In some runs, no peak fraction appeared and the lectin activity was eluted more or less as a continuum from high to low molecular weight.

4.2 Elution in the Presence of Urea:

In order to reduce the interaction between the protein and the column matrix which could be responsible

FIGURE 9: Purified cleavage extract containing 0.49×10^6 HU and 0.23 mg protein was eluted in SPBS. 0.031×10^6 HU were recovered which represents 6.3% of the initial activity. The apparent molecular weight of the peak fraction (arrow) is 440,000.


Titer (H.U.)
0
10
20
30
40
50
60
70
Fraction Number
15
20
25
30
35
40
45
-669,000
-440,000
-232,000
-67,000
-45,000

for the trailing seen in Figure 9, the lectin was eluted with urea (2 M) in the elution buffer. Figure 10 shows the elution profile obtained when 0.12 mg of protein from purified gastrula extract was eluted in SPBS with urea (2 M). The arrows point to the presence of three peak fractions at molecular weights of 207,000, 148,000 and 97,000. In all, eight experiments were performed under these conditions. Peaks of lectin activity eluted from the column ranged from a low of 2,500 to a high of 374,000 molecular weight. No two experiments showed the same elution profile. Some trailing of the lectin activity into the small molecular weight range was again noticed.

4.3 Elution in the Presence of Lactose:

The elution profile for crude gastrula extract in the presence of lactose (0.1 M) is shown in Figure 11. Gel filtration under these conditions resulted in the appearance of a single peak of activity with an apparent molecular weight of 495,000. The results obtained using crude and purified material from other stages were similar. Six experiments conducted under these conditions gave a mean molecular weight of the peak fraction of 490,794. The estimated molecular weight in the presence of lactose agrees with the value recently reported by Roberson and

FIGURE 10: Purified gastrula extract containing 0.34×10^6 HU and 0.12 mg protein was eluted with SPBS + urea (2 M). 0.094×10^6 HU (27.6%) were recovered. The apparent molecular weights of the peak fractions (arrows) are; 207,000, 148,000 and 97,000.

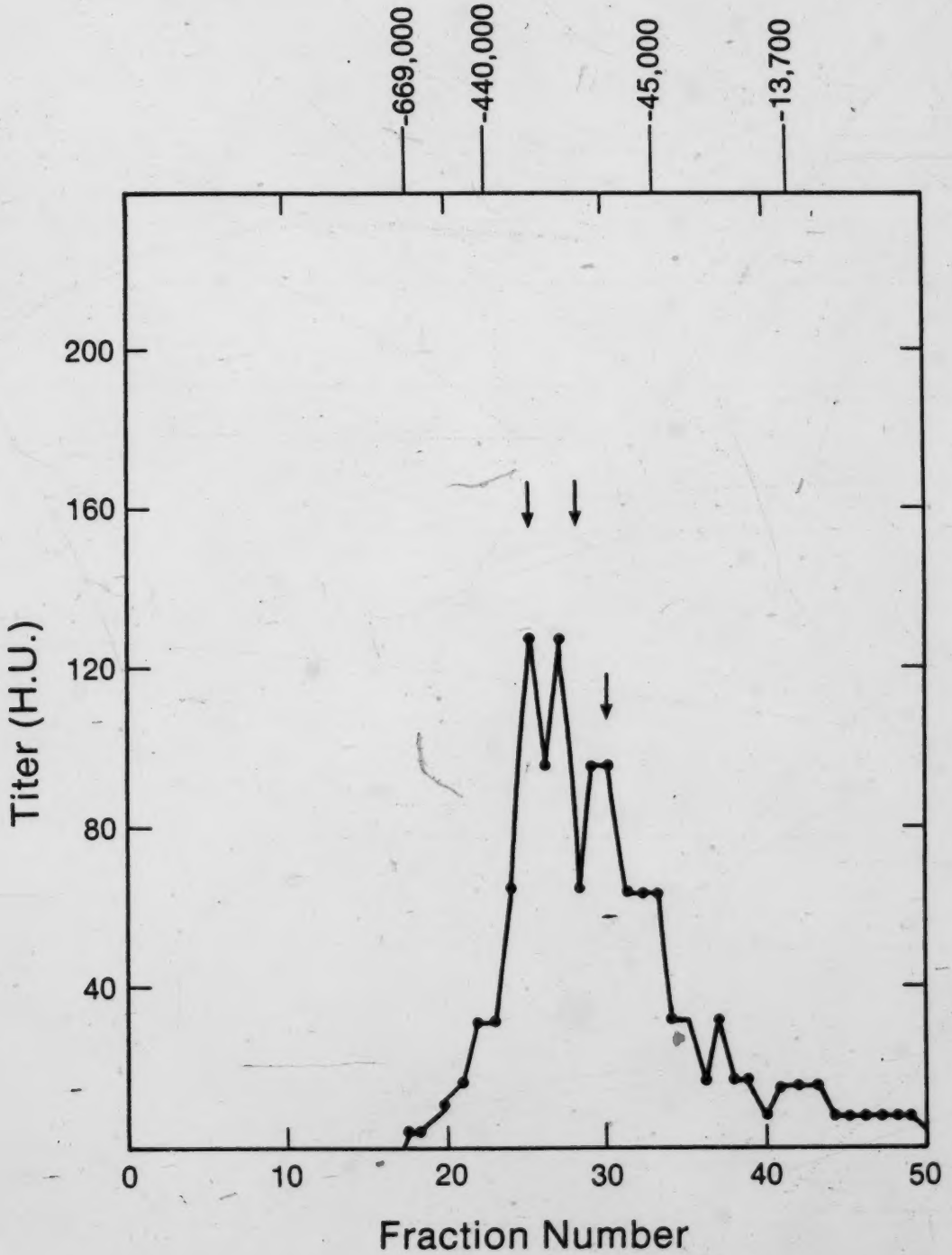
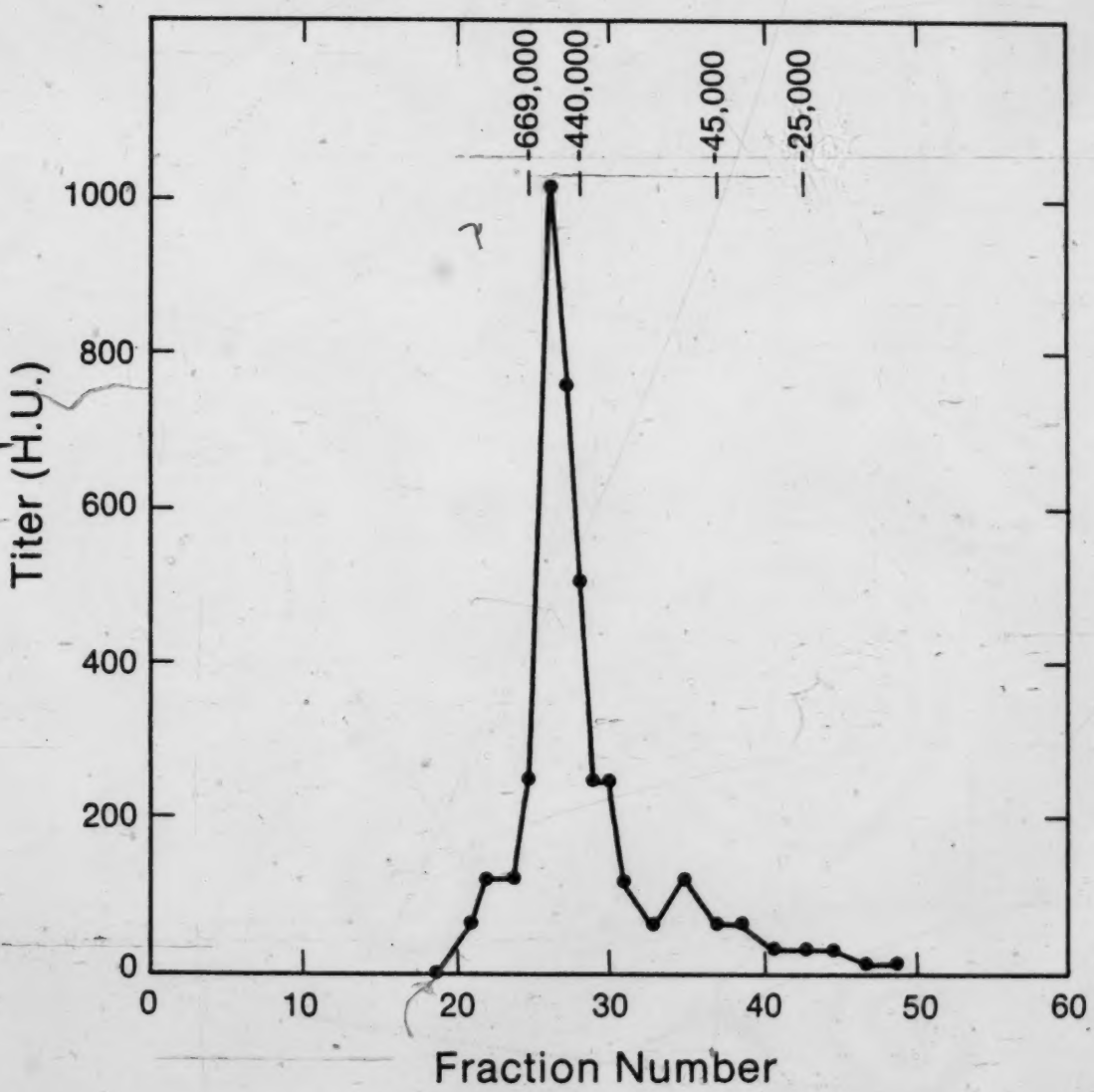

-669,000
-440,000
-45,000
-13,700
200
160
120
80
40
Titer (H.U.)
0
10
20
30
40
50
Fraction Number

FIGURE 11: Crude gastrula extract eluted with SPBS containing lactose (0.1 M). 1.64×10^5 HU and 1.4 mg protein was added to the column and 0.39×10^6 HU (24%) was recovered. The apparent molecular weight of the peak fraction is 495,000.


669,000
440,000
45,000
25,000
1000
800
600
400
200
0
Titer (H.U.)
0
10
20
30
40
50
60
Fraction Number

Barondes (1982) for lectin extracted from Xenopus oocytes.

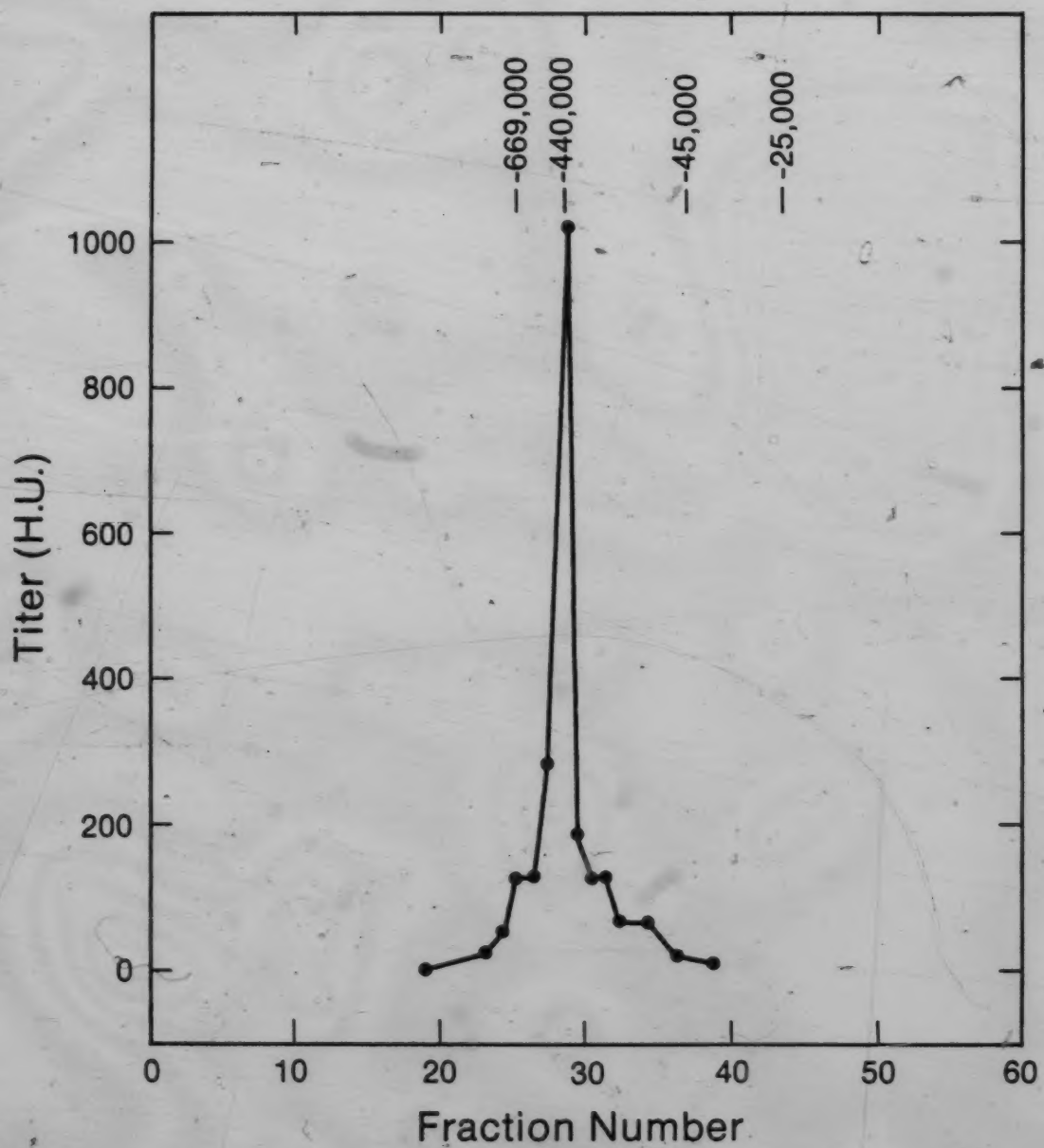
4.4 Gel Filtration of Chloroform-Methanol Treated Extracts:

Figure 12 shows the elution profile obtained with crude lectin from gastrula embryos treated with chloroform-methanol and eluted from the column with SPBS containing lactose (0.1 M). As in the previous figure, a single peak of activity was obtained. The apparent molecular weight of this fraction was 371,000. This value is lower than the molecular weight obtained when untreated extract from the same clutch of embryos was eluted under the same conditions (Figure 11). It is evident that some material associated with the lectin molecule or included in the lectin aggregates is removed by this treatment. This experiment was not repeated using extracts from other developmental stages.

5. MOLECULAR PROPERTIES OF THE XENOPUS LECTIN

Several biochemical procedures were employed to determine some of the molecular properties of the Xenopus lectin. Firstly, the molecular weight of the dissociated and denatured polypeptide chains was estimated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS). Secondly, the isoelectric

FIGURE 12: Crude gastrula extract treated with chloroform-methanol and eluted under the same conditions as Figure 11. In this experiment 0.5×10^6 HU were added to the column (0.25 mg protein). 0.2×10^6 HU were collected (38.8%). The specific activity of the original sample was 2×10^6 HU/mg and that of the peak fraction was 2.93 x 10HU/mg, representing an increase in specific activity of 1.5X. The peak fraction has an apparent molecular weight of 371,000.


669,000
440,000
45,000
25,000
1000
800
600
400
200
0
Titer (H.U.)
0
10
20
30
40
50
60
Fraction Number

point of the polypeptide was examined by isoelectric focussing in thin layer polyacrylamide gels. Thirdly, the amino acid composition was estimated.

5.1 Polyacrylamide Gel Electrophoresis (PAGE):

PAGE was carried out in 10% flat bed gels in the presence of SDS according to the procedure of Laemmli (1970) as described in Materials and Methods. All of the samples loaded on the gels were solubilized either in sample buffer containing SDS, urea and mercaptoethanol or by following the methods of Kawasaki and Ashwell (1976). The patterns of the bands obtained depended to a large extent on the method used to purify the protein samples.

5.1.1 Crude Soluble Protein Extract.

Crude extracts of Xenopus embryos at all stages showed a number of protein bands ranging from low to high molecular weight. Crude samples untreated with chloroform-methanol show a pattern which is complicated by diffusely stained areas (Figure 13A). Such areas may be due to glycoproteins or lipids which do not migrate as a discrete band under these conditions (Dzandu et al, 1984). Crude protein extracts treated with chloroform-methanol show fewer bands than untreated material (Figure 13B). In two

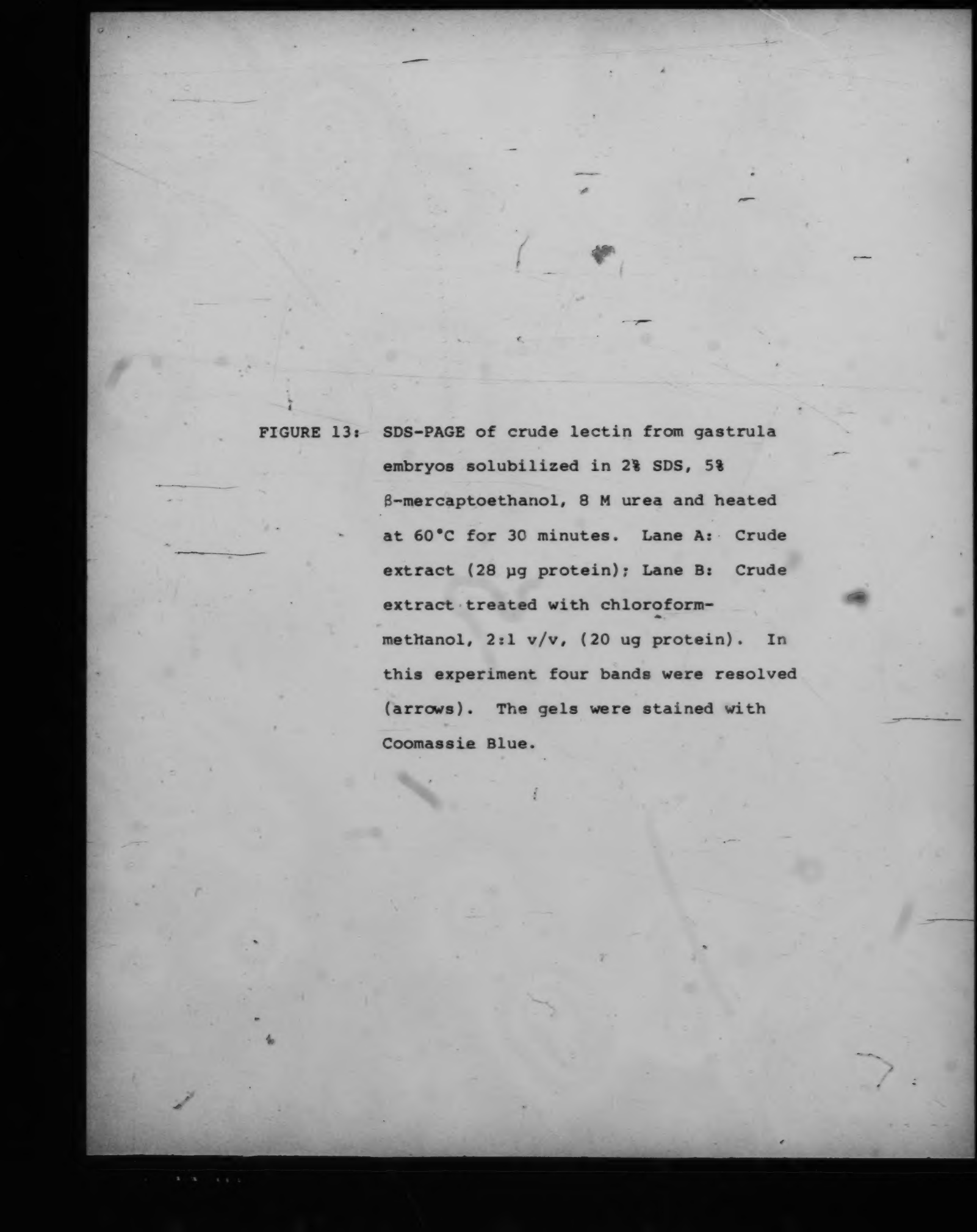


FIGURE 13: SDS-PAGE of crude lectin from gastrula embryos solubilized in 2% SDS, 5% β-mercaptoethanol, 8 M urea and heated at 60°C for 30 minutes. Lane A: Crude extract (28 µg protein); Lane B: Crude extract treated with chloroform-methanol, 2:1 v/v, (20 ug protein). In this experiment four bands were resolved (arrows). The gels were stained with Coomassie Blue.

A
B

experiments, these bands ranged in number from 4 to 8 and in molecular weight from 10,000 to 100,000. The diffusely staining material characteristic of untreated samples was not noticeable in chloroform-methanol treated samples. In both cases, some material remained in the wells and did not enter the gels.

5.1.2 αGal IA-Purified Material.

Lectin from all stages purified on αGal IA gave rise to a pattern of bands which was not consistent between preparations. In all, nine groups of proteins can be distinguished according to their molecular weights as follows; A(10-14,000); B(20-24,000); C(34,000); D(43-48,000); E(51-55,000); F(61-64,000); G(70,000); H(90,95,000); I(100,000). Typical patterns obtained from samples of different embryonic stages are shown in Figure 14. It can be seen from this figure that considerable discrepancies exist between samples and the differences were not consistent between or within stages. The frequency of occurrence of these bands found in many samples from the different developmental stages is shown in Table 11. Band D (frequency 0.98) was found most consistently and was present in all stages. This

FIGURE 14: SDS-PAGE of αDGal-1A purified lectin. Samples were solubilized in 2% SDS, 5% β-mercaptoethanol, 8 M urea and heated at 60°C for 30 min. Coomassie brilliant blue stain. Bands are labelled arbitrarily according to their range of molecular weights: A(10-14,000), B(20-24,000), C(34,000), D(43-48,000), E(51-55,000), F(61-64,000), G(70,000), H(90-95,000) and I(100,000). Lane 1, cleavage and early blastula (st. 8,9); Lane 2, blastula (st. 8,9); Lane 3, midgastrula (st. 11,12); Lane 4, late gastrula (st. 13, yolk plug); Lane 5, blastula (st. 6,7,8); Lane 6, standards; BSA (67,000), ovalbumin (45,000), chymotrypsinogen (25,000), ribonuclease (13,000); Lane 7, BSA standard.

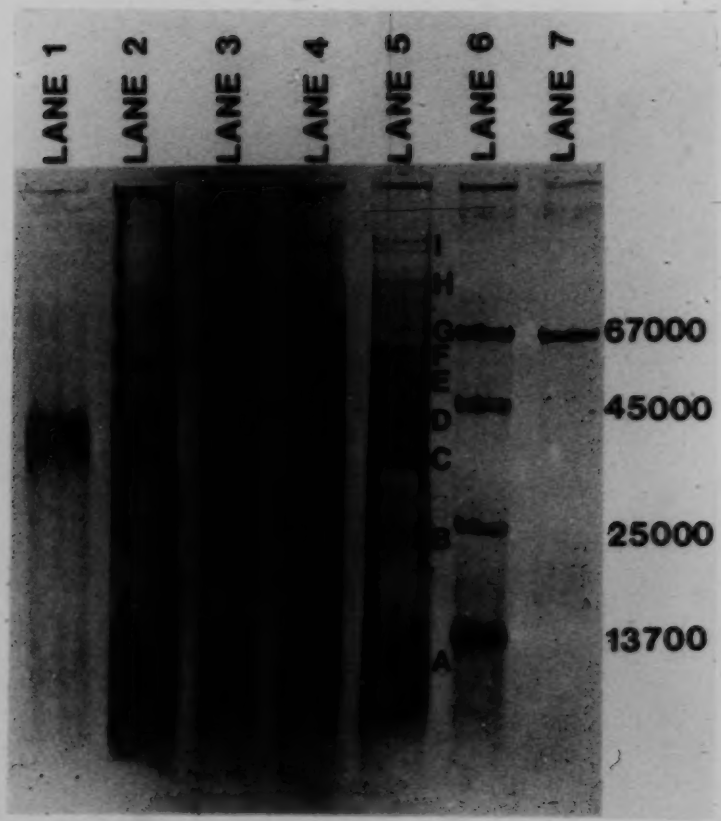

LANE 1
LANE 2
LANE 3
LANE 4
LANE 5
LANE 6
LANE 7
67000
45000
25000
13700
H
G
F
E
D
C
B
A

TABLE 11: The frequency of occurrence of lectin bands obtained by SDS-polyacrylamide gel electrophoresis at different stages of development. Values represent the number of times a given band appeared divided by the number of samples run for any given stage.

BAND	MOLECULAR WEIGHT	STAGE				OVERALL FREQUENCY OF OCCURRENCE
		CLEAVAGE (4)*	BLASTULA (10)	GASTRULA (6)	NEURULA (3)	
A	10-14,000	0.75	0.5	0.5	1.0	0.69
B	20-24,000	0.25	0.5	0.63	0	0.35
C	34,000	0.25	0.7	0.75	0.3	0.35
D	43-48,000	1.0	0.9	1.0	1.0	0.98
E	51-55,000	0.5	0.3	0.75	0.33	0.47
F	61-64,000	0.5	0.6	0.5	0.66	0.57
G	70,000	0	0.4	0.75	0.66	0.45
H	90-95,000	0	0.3	0.5	0.33	0.28
I	100,000	0.25	0.2	0.13	0.33	0.23

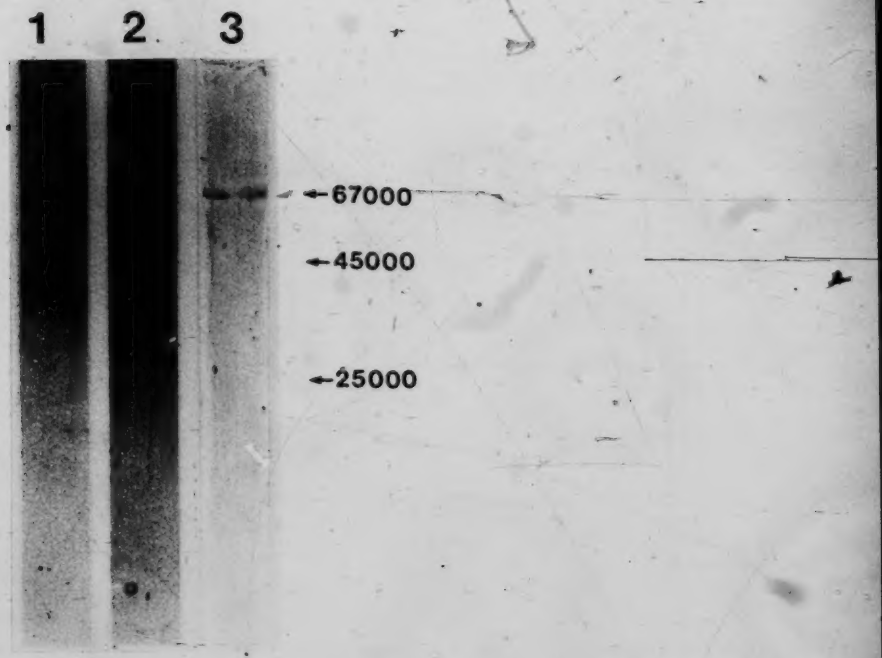
*Numbers in parenthesis refer to the number of purifications, each obtained from a different clutch of embryos.

band corresponds to the two subunits of molecular weight 43,000 and 45,000 reported by Roberson and Barondes (1982) for the lectin from Xenopus oocytes. Seven bands were obtained from cleavage extracts. Besides D, band A was the most frequent, while bands G and H were absent. Blastula extracts showed all nine bands, the most frequent being bands D and C. In gastrula extracts the most conspicuous bands were D, C, E and G. Under our conditions, band B which showed a relatively high frequency at gastrula (0.63) disappears from neurula stage extracts.

5.1.3 APL-Sepharose Purified Lectin.

The reason for the high variability and the high number of bands observed in samples purified with αGal IA is not fully understood. For comparison, lectin purified by affinity chromatography on APL-Sepharose was also run on PAGE under the same conditions used for the αGal-IA purified material. Gastrula lectin purified on APL-Sepharose gave rise to a distinct band at 64,000 and a broad band ranging from 37,000 to 48,000 (Figure 15, lane 1). The latter band corresponds to the band D which occurred with the highest frequency in samples purified with αGal IA.

FIGURE 15: SDS-PAGE of embryonic lectin. Samples were solubilized in sample buffer containing SDS (2%), β-mercaptoethanol (5%), urea (8 M) and heated at 60°C for 30 minutes. Lane 1: Gastrula lectin purified on APL-Sepharose gave rise to a distinct band at 64,000 and a broad band at 37,700 to 48,000. Lane 2: Gastrula lectin treated with chloroform-methanol and purified on APL-Sepharose. One band at 64,000 is evident. Lane 3: Early blastula lectin treated with chloroform-methanol and purified on αGal IA gave rise to a single band at 65,000. 10-20 μg of purified lectin was added to each well. These gels are stained with silver. Molecular weight markers are indicated by arrows.


1
2
3
67000
45000
25000

5.1.4 Chloroform-Methanol Treated Lectin.

When purified Xenopus from any embryonic stage was treated with chloroform-methanol before solubilizing in sample buffer and run on SDS-PAGE, a single band was observed. Figure 15, lane 2, shows gastrula lectin treated with chloroform-methanol and subsequently purified on APL-Sepharose. One band at 64,000 molecular weight is evident. Similarly, blastula lectin treated with chloroform-methanol and purified with αGal IA gives rise to a single band at 65,000 molecular weight (Figure 15, lane 3). From five separate determinations, the molecular weight of this protein which was cleaned of associated material by chloroform-methanol treatment was 65,500 ± 2,780. Similar values were obtained from preparations of cleavage and gastrula lectin. One determination of chloroform-methanol treated purified oocyte lectin resulted in a single band at 72,443 molecular weight.

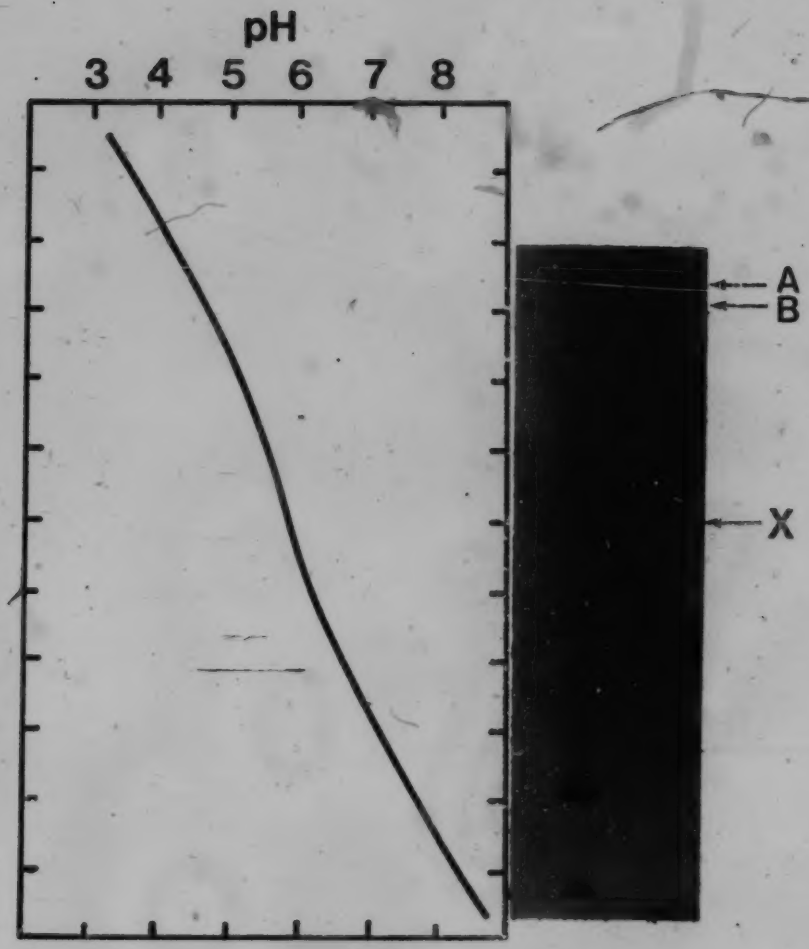
5.2 Isoelectric Focussing:

Single bands on SDS-PAGE may actually consist of more than one component which can be resolved by isoelectric focussing. To determine if this was the case with the lectin from Xenopus embryos, isoelectric focussing experi-

ments were carried out using the procedure of Cook (1976) as described in Materials and Methods. All experiments were performed on lectin samples purified with αGal IA. When lectin from blastula embryos was focussed, two bands were resolved at pI 4.3 and 4.5 (Figure 16). A similar result was obtained with lectin from gastrula embryos. The pH range shown by the embryonic lectin under these conditions is similar to the range in pI values reported by Roberson and Barondes (1982) for the lectin from Xenopus oocytes, although four bands were resolved under the conditions used by these authors. When the lectin sample used for Figure 16 was subjected to SDS-PAGE it gave rise to eight bands. Presumably, some of these bands are due to material in the sample which is solubilized by the SDS in the sample buffer and the dissociating conditions used for PAGE, allowing this material to enter the gel. Under the milder conditions used for isoelectric focussing including the TRIS-glycine buffer, only the water soluble material entered the gel.

Isoelectric focussing of chloroform-methanol treated lectin resulted in two bands similar to those shown in Figure 16. It appears that the two components that separate at pI 4.3 and 4.5 are derived from a single protein band of 65,500 molecular weight observed in the PAGE samples of chloroform-methanol treated extracts.

FIGURE 16: Isoelectric focussing of purified lectin from blastula embryos (stage 8,9). The sample (50 µg) was dissolved in glycine (1%) in distilled water and was focussed at 1200 V and 10 mA for 20 hours over a pH range of 3.5-10. The graph on the left shows the pH gradient in the gel measured with a surface electrode. The bands produced by the standards (lane 1) and the sample (lane 2) are shown in the photograph on the right. The point of application of the samples is marked 'X' and the two bands produced by the lectin sample are marked A (pI 4.3) and B (pI 4.5). The gel was stained with Coomassie blue. The standards from acid to basic pH are: soybean trypsin inhibitor (pI 4.55), β-lactoglobulin (pI 5.2), bovine carbonic anhydrase (pI 5.85), human carbonic anhydrase (pI 6.55), horse myoglobin (pI 6.85 and 7.35), lentil lectins (pI 8.15, 8.45, 8.65).


pH
3 4 5 6 7 8
A
B
X

5.8 Amino Acid Composition:

The amino acid composition of lectin treated with chloroform-methanol and purified with αGal IA is shown in Table 12. No hexosamines were detected in this analysis, a finding which is considerably different from the results reported by Roberson and Barondes (1982) for the Xenopus oocyte lectin. This lack of carbohydrate is confirmed by the absence of staining of purified chloroform-methanol treated lectin with periodic acid Schiff stain on PAGE gels.

6. THE LOCALIZATION OF THE XENOPUS EMBRYONIC LECTIN

The experiments described in this section were designed to investigate the location of the lectin in early embryos of Xenopus laevis. Initially, a polyclonal antibody was produced in rabbits against IA-affinity purified lectin that had been treated with chloroform-methanol (See Materials and Methods, Section 10.2). This antiserum was characterized by agar diffusion, by its ability to inhibit lectin-induced haemagglutination, and by protein immunoblotting. These antibodies were subsequently used to detect the lectin in sections of early Xenopus embryos using immunofluorescence.

TABLE 12: The amino acid composition of the Xenopus embryonic lectin. Chloroform-methanol treated, α-Gal IA purified lectin from gastrula embryos was hydrolysed in boiling 6N HCl and 1% phenol for 24 hours and analysed in a Durram D-500 amino acid analyser. ND = not determined.

AMINO ACID	RESIDUES/65,000 MOLECULAR WEIGHT
Asp	51
Thr	26
Ser	80
Glu	66
Gly	150
Ala	67
Val	25
Ile	15
Leu	27
Tyr	7
Phe	9
His	12
Lys	18
Arg	11
Pro	19
Met	2
Cys	ND
Try	ND

6.1 Agar Diffusion Tests:

Agar diffusion experiments was one method used to test for the presence of specific antibodies in the antiserum. In one experiment a single precipitin line was seen. In this plate, antiserum was added to the center well and crude Xenopus lectin extract was added in increasing dilutions to the external wells. The original concentration of the crude lectin was 0.2 µg/µl and the final dilution of the lectin extract showing a precipitin line was 1:8. A second plate with antiserum in the center well and α-Gal IA purified, chloroform-methanol treated lectin from gastrula embryos diluted in the outer wells gave similar results. In this case, the original concentration of the lectin was 0.09 µg/µl and the final dilution of the lectin sample showing a precipitin line was 1:32.

6.2 The Inhibition of Lectin-Induced Agglutination by Antiserum:

The ability of the polyclonal antiserum to interfere with the lectin-mediated agglutination of rabbit erythrocytes was examined. Antiserum of increasing dilutions was incubated overnight with equal volumes of crude lectin extract from gastrula embryos. After centrifugation to remove any precipitate formed, the supernatant

was tested for haemagglutinating activity. The results of this experiment are shown in Table 13. There is a differential inhibition of lectin activity by the antibody preparation. Control serum inhibited 50% of the agglutinating activity at a dilution of 1:10 and had no inhibitory activity at dilutions of 1:100. In contrast, the antiserum inhibited 64 HU at dilutions of 1:100 and 1:1,000. It is apparent that the polyclonal antiserum contained antibodies which interacted with the lectin molecule in the extract.

6.3 Protein Immunoblotting:

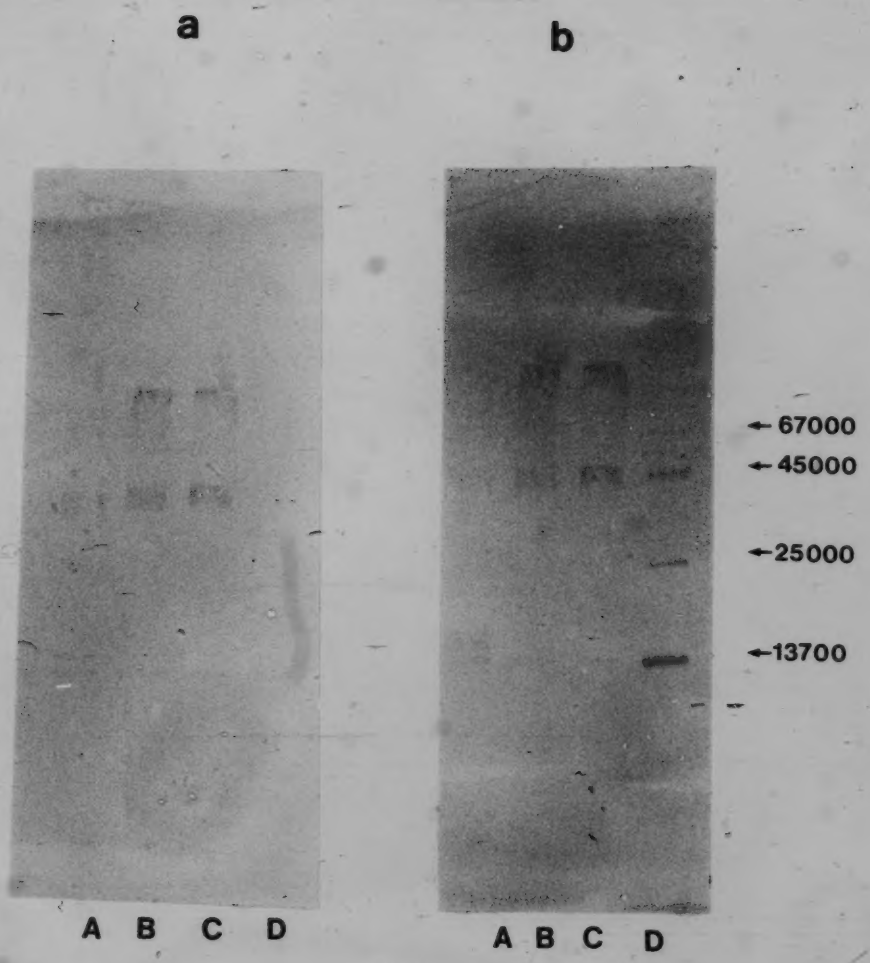
Crude and purified lectin samples were electrophoresed on PAGE in duplicate and transferred to nitrocellulose paper. Blots stained with anti-lectin antiserum are shown in Figure 17a. The same blot subsequently stained with Amido Black is shown in Figure 17b. In both photographs, Lane A is purified lectin, Lanes B and C are crude lectin samples, and Lane D is the standards. In both purified and crude preparations, anti-lectin antiserum stains a broad band of approximately 43,000 to 47,000 molecular weight. The 65,500 lectin protein is not stained by this procedure.

Purified protein dissolved in SPBS and dotted directly onto nitrocellulose paper followed by staining

TABLE 13: Inhibition of agglutination by antiserum. A, Control lectin mixed 1:1 (v/v) with SPBS. B, Lectin mixed 1:1 (v/v) with increasing dilutions of control serum in SPBS. C, Lectin mixed 1:1 (v/v) with increasing dilutions of anti-lectin antiserum in SPBS. Tubes were shaken briefly, incubated overnight at 4°C and centrifuged at 9.000 x g for 10 minutes before testing. The results of two inhibition tests are averaged here (the anti-lectin antiserum diluted 1:1,000 gave a titer of 0 in one test and 2 in the second). Control serum was taken from the rabbit before injection of the lectin antigen.

	SAMPLE	TITER
A	Control (lectin extract 1:1, SPBS)	64
B	Lectin + Control Serum	0
	Lectin + Control Serum (1:1)	0
	Lectin + Control Serum (1:10)	32
	Lectin + Control Serum (1:100)	64
C	Lectin + Anti-lectin Antiserum	0
	Lectin + Anti-lectin Antiserum (1:1)	0
	Lectin + Anti-lectin Antiserum (1:10)	0
	Lectin + Anti-lectin Antiserum (1:100)	0
	Lectin + Anti-lectin Antiserum (1:1,000)	1
	Lectin + Anti-lectin Antiserum (1:10,000)	64

FIGURE 17: Protein immunoblotting of Xenopus embryonic lectin. Samples were electrophoresed on PAGE gels and bands were transferred to nitrocellulose paper. (a) shows the gel stained with anti-lectin antiserum, (b) shows the same gel stained with Amido black. In both photographs, Lane A is purified lectin, Lanes B and C are crude lectin and Lane D is protein standards. Molecular weights of the standards are indicated.


a
b
67000
45000
25000
13700
A B C D
A B C D

with anti-lectin immune serum shows positive staining (Figure 18a). No staining of this material is shown with pre-immune serum (Figure 18b). The sample used in this experiment showed a single band at 65,500 molecular weight on PAGE, however, when this sample was transferred to nitrocellulose paper and stained with immune serum, no staining was detected. The same sample used for PAGE, dotted directly onto nitrocellulose paper, did not stain.

6.4 Immunohistochemistry:

6.4.1 Fluorescent Staining.

Cleavage embryos (stage 1½-5) and blastula embryos (stage 7-8) were fixed, sectioned, labelled with immune or pre-immune serum and stained with FITC-goat anti-rabbit antibodies, as described in Materials and Methods. A control section treated with pre-immune serum is shown in Figure 19. Some background autofluorescence, particularly in the yolk granules, is evident in this section. Such autofluorescence is usually prevalent in yolky embryos. Two illustrations of the organization of stained material in early Xenopus embryos are presented here. Figure 20a is a phase contrast photomicrograph of a cleavage furrow present in a

FIGURE 18: Dot immunoblotting of Xenopus embryonic lectin. The lectin sample used in this experiment appeared as a single band of 65,000 molecular weight on SDS-PAGE gels. This sample was also used for amino acid analysis, the results of which are shown in Table 12. (a) shows anti-lectin antiserum staining of purified gastrula lectin dotted directly onto nitrocellulose paper. (b) shows the same sample treated with pre-immune serum.

L = Lectin, 12.5 ng

SA = Serum Albumin, 300 µg

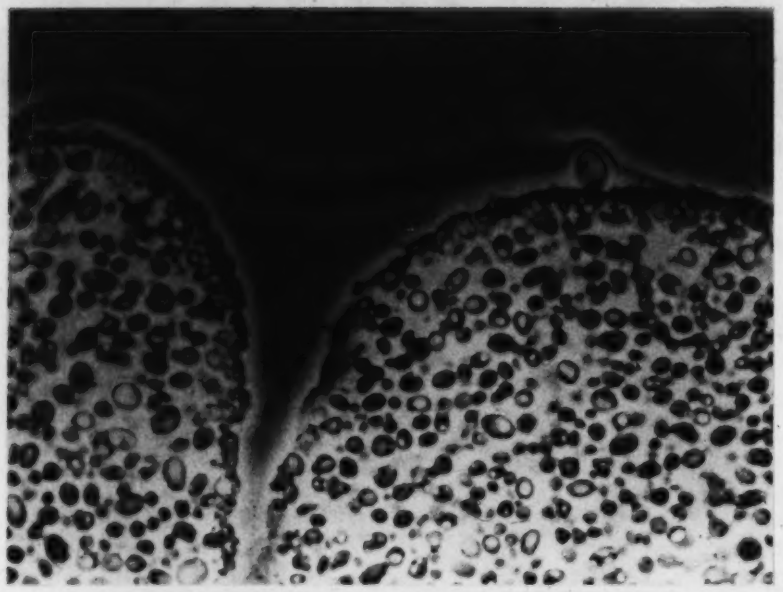
HH 105
C
A
b
a

FIGURE 19: Control section of a stage 5 Xenopus embryo. This embryo was fixed in Bouin's fixative, embedded in polyethylene glycol and the section was stained with pre-immune serum. This section, observed with fluorescence microscopy, shows some autofluorescence, particularly associated with the yolk granules.

50 μm

FIGURE 20: Cleavage furrow of a stage 5 embryo which was fixed in paraformaldehyde, sectioned and stained with anti-lectin antiserum. This section was viewed with phase-contrast optics (a) and fluorescence optics (b). Extracellular material lying on the surface of the blastomere under the vitelline membrane and deeper in the cleavage furrows appears stained.

a



b

50 μm

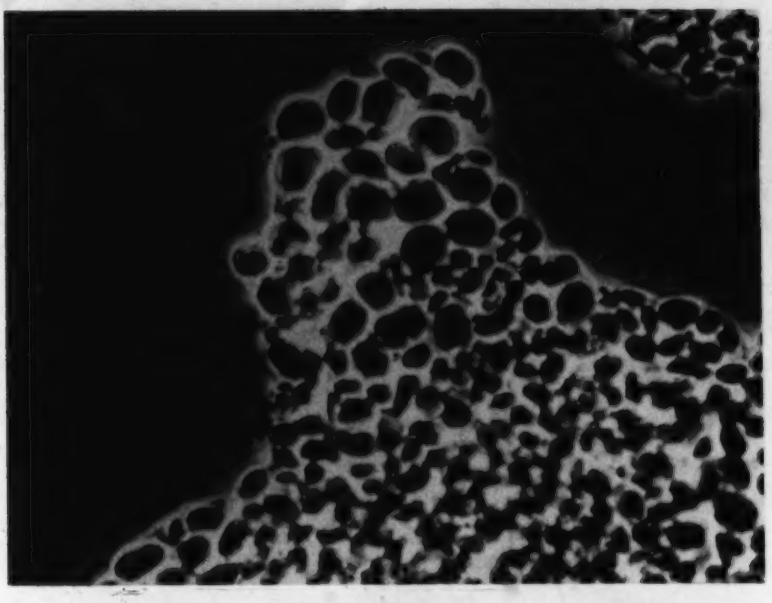
stage 5 embryo. The vitelline membrane (arrow) is prominent in this section. Figure 20b is the same section viewed with fluorescence mircoscopy. Anti-lectin antibodies stain the external surface of the blastomeres, material located between the cells deeper in the cleavage furrows, and loose material lying above the cell surface but underneath the vitelline membrane. In this section, the vitelline membrane does not appear to be stained. However, in other experiments the vitelline membrane was also stained by the antiserum. This discrepancy could be due to the differential cross-linking of material by fixation.

Figure 21 is a section showing a lobopodium extension facing the blastocoele cavity. The phase contrast micrograph (Figure 21a) shows the many yolk granules typical of these cells. Under fluorescence microscopy (Figure 21b), the intracellular material is labelled (arrow). This material may be in the process of being secreted or phagocytosed.

Figure 22 shows fluorescent staining of a whole embryo at the early blastula stage. In this section, anti-lectin antibodies stain the vitelline membrane and extracellular material located under the vitelline membrane in those areas where the vitelline

FIGURE 21: Lobopodium of a cell from a stage 5 embryo, facing the blastocoele cavity. This embryo was frozen in OCT before sectioning. (a) shows the appearance of the cell extension using phase contrast optics, and (b) shows the same view using fluorescence optics. Positive staining is apparent in yolk-free cytoplasm adjacent to the blastocoele cavity (arrow).

a



b

50 μm

FIGURE 22: Fluorescence microscopy of a stage 9 embryo which was fixed in paraformaldehyde and embedded in polyethylene glycol prior to sectioning. Areas of staining are indicated by arrows and include the vitelline membrane (small arrow), extracellular material lying under the vitelline membrane, and intercellular deposits (large arrow).

200 μm

membrane remains intact. Staining is also seen along the roof of the blastocoele cavity, in yolky endoderm cells below the blastocoele cavity, and some discrete deposits in vegetal hemisphere cells. It was not possible to determine if yolk granules contributed to the staining observed here, although other sections showed staining associated with the periphery of some yolk granules.

6.4.2 Glucose Oxidase-Antiglucose Oxidase Staining.

The location of the lectin was also determined by treatment of sectioned material with glucose oxidase-antiglucose oxidase. Figure 23a shows a control section through surface blastomeres of a stage 5 embryo. No vitelline membrane is present. Some dark granules representing either pigment granules or areas of endogenous glucose oxidase activity are shown at the surface of these cells. Figure 23b is a section of surface blastomeres stained with anti-lectin antiserum. Heavy deposits appear on the surface of these cells and within the cleavage furrows.

Deeper blastomeres of embryos at the same stage are shown in Figure 24. Figure 24a shows a section

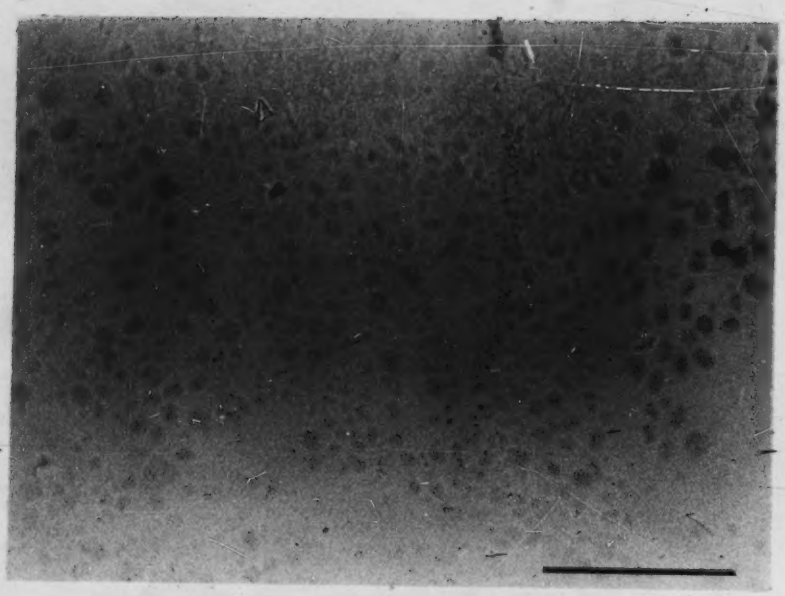
FIGURE 23: Glucose oxidase-antiglucose oxidase staining of surface blastomeres of a stage 5 embryo fixed in Bouin's fixative and embedded in polyethylene glycol. Some staining is seen in the control section treated with pre-immune serum (a), but heavier deposits are evident in the material treated with anti-lectin antiserum (b). Staining is enhanced along the external surface of the blastomeres and in the cleavage furrow between adjacent blastomeres.

a

b

FIGURE 24: Glucose oxidase-antiglucose oxidase staining of deeper blastomeres of a stage 5 embryo treated as in Figure 23. The control section (a) shows light staining. Heavier staining is seen in the section treated with anti-lectin antiserum (b), particularly along the membranes of these cells and between adjacent cells.

a



b

treated with control serum. The section in Figure 22b was treated with immune serum and shows an increased amount of staining between adjacent membranes of deeper cells.

7. DETECTION OF A POSSIBLE ENDOGENOUS LECTIN RECEPTOR

The membrane-enriched pellet obtained by centrifugation of the embryonic homogenate was further examined for the presence of a possible membrane-associated endogenous receptor for the lectin activity. The pellet was extracted with 2% Triton X-100, stirred overnight at 4°C, centrifuged at 84,000 x g for 60 minutes and a 2 ml sample was applied to a Sephacryl S-300 column. Fractions were eluted with 0.05% Triton X-100 in SPBS. Each fraction was dialysed separately to remove the detergent and tested for the ability to inhibit the agglutination of rabbit erythrocytes by crude gastrula lectin extract. The results are shown in Figure 25. A single peak at approximately 140,000 molecular weight inhibited 128 HU of lectin activity. No other fraction showed any inhibitory activity.

This inhibiting fraction was lyophilized, solubilized in sample buffer and applied to SDS-PAGE. The results are shown in Figure 26. Lane 1 shows the resolution of two bands in a gel stained in Coomassie blue (arrows). One

FIGURE 25: The inhibition of lectin activity by the Triton-solubilized membrane pellet from Xenopus embryos. Fractions (3.5 ml) were eluted from a column (2.5 x 40 cm) of Sephacryl S-300, dialysed separately against SPBS and tested in an agglutination inhibition test. This assay included 25 µl serially diluted lectin, 25 µl SPBS for control wells or 25 µl fractionated, solubilized membrane material for test wells, 25 µl BSA (1%) and 25 µl rabbit erythrocytes (4% solution). Control wells had a titer of 128.

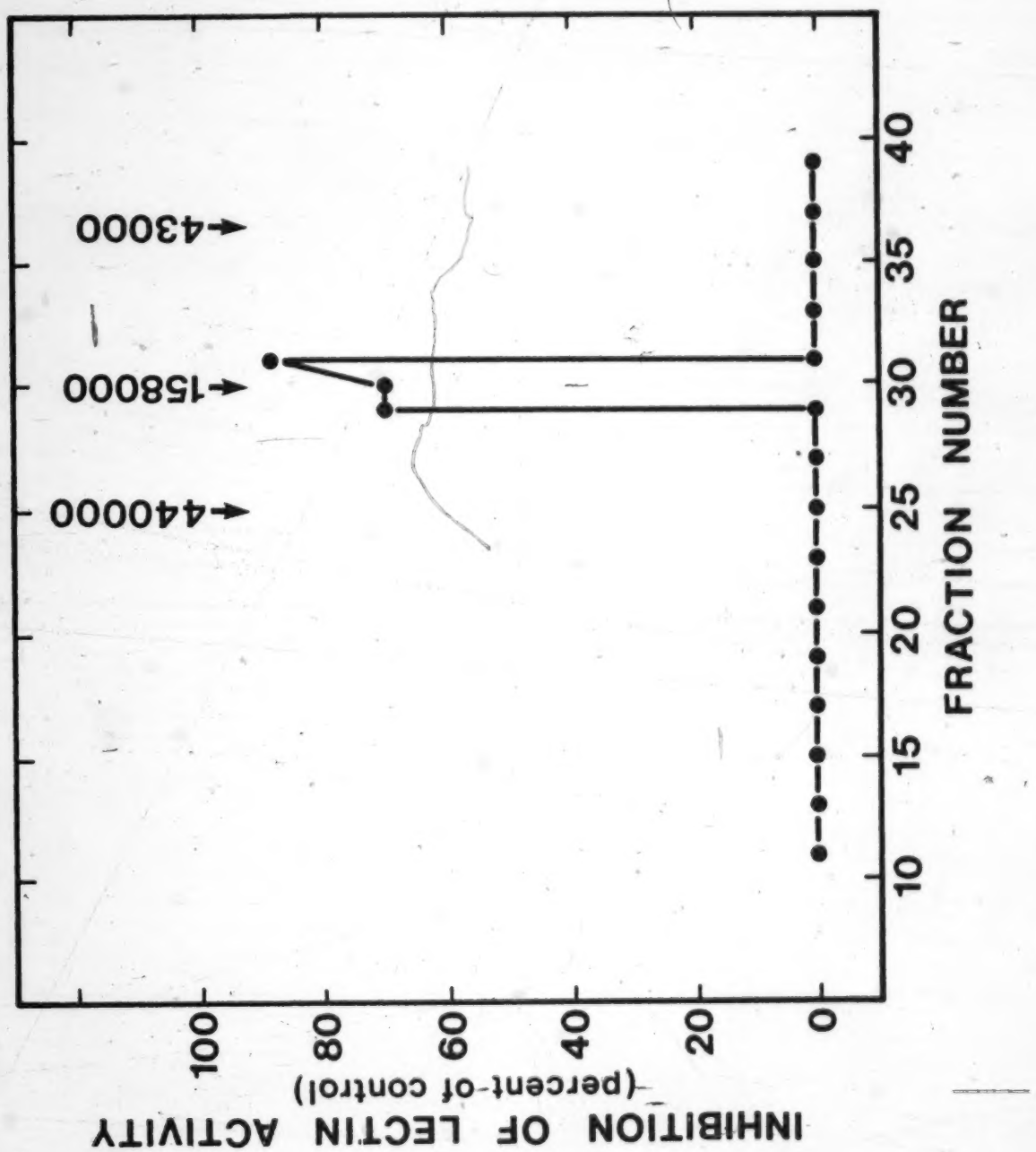
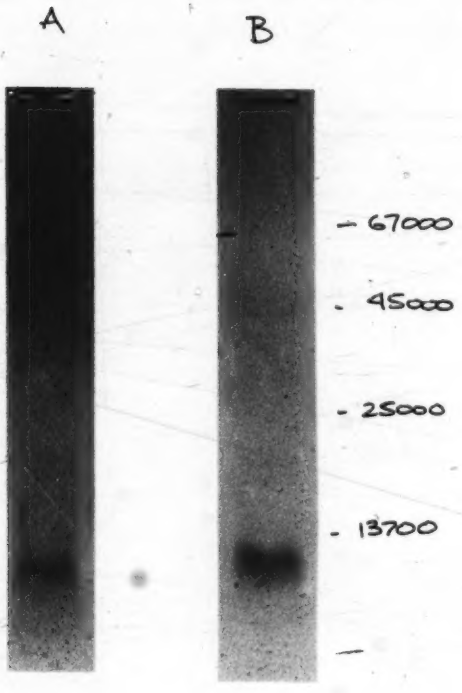

INHIBITION OF LECTIN ACTIVITY
(percent of control)
0
20
40
60
80
100
10
15
20
25
30
35
40
FRACTION NUMBER
440000
158000
43000

FIGURE 26: SDS-PAGE of Triton-solubilized membrane pellet. Samples were run in duplicate on a single gel. Lane A shows two bands resolved in Coomassie blue stain; one at 65,000 and one at 9,700-11,000 molecular weight. Lane B shows a single band resolved by PAS at 9,700-11,000 molecular weight. The molecular weights of the bands produced by the standards are indicated. Samples and standards were solubilized in sample buffer containing SDS (2%), mercaptoethanol (5%) and urea (8 M) and heated at 60°C for 30 minutes.


A
B
67000
45000
25000
13700

band has an apparent molecular weight of 65,000 and the second is a broad band between 9,700 and 11,000 molecular weight. Lane 2 shows the same samples run on the same gel as in lane 1 but stained with periodic acid Schiff (PAS) stain for carbohydrates. Only one band is stained, corresponding to the broad band at 9,700-11,000 molecular weight. It is possible that the 65,000 molecular weight band appearing in lane 1 is the Xenopus lectin and the endogenous receptor is a small molecular weight glycoprotein.

DISCUSSION:

1. THE OCCURRENCE OF AN ENDOGENOUS LECTIN IN XENOPUS EMBRYOS

Cells of the amphibian embryo just prior to gastrulation can be agglutinated by treatment with the plant lectins WGA, RCA, Con A and to a lesser extent SBA (Fraser and Zalik, 1977), indicating that surface glycoconjugates with exposed N-acetylglucosamine, galactose, N-acetylgalactosamine and glucose or mannose residues are acting as receptors for these lectins. This study, together with the fact that an endogenous β-galactose binding lectin had been reported in gastrulating chick embryos (Cook et al, 1979) encouraged the present investigation into the occurrence of endogenous lectins in Xenopus embryos. This study reports on the presence and the partial characterization of an endogenous galactose binding lectin in Xenopus embryos at stages prior to and during gastrulation. The role of this lectin may be to modify the cell surface during these early developmental stages. When this work began, no report existed in the literature of endogenous soluble lectins in amphibian embryos.

Soluble extracts prepared from Xenopus embryos agglutinate trypsinized rabbit erythrocytes which have been fixed with glutaraldehyde or stabilized by treatment with azide and PMSF. The specific activity of the Xenopus embryonic lectin is higher than specific activities reported for other embryonic or adult tissues. This indicates either that this lectin shows extreme potency or that it is more abundant in embryos of this species. Unfortunately, the specific activities of soluble lectin in early embryonic stages of other emphibian species have not been reported so it is impossible at this time to determine if lectins of this type are a general phenomena in amphibian embryonic development or if this situation is peculiar to Xenopus. Lectin activity with high titer is also detectable in extracts of Xenopus ovarian tissue and in fluid withdrawn from the blastocoele cavity of stage 7 embryos. Early work on the characterization of the Xenopus embryonic lectin has been published previously (Harris and Zalik, 1982; 1985).

In this study, the presence of lactose in the extraction medium was found to release the maximum amount of lectin from the homogenate. The presence of competing saccharides during extraction may prevent binding of the lectin to other cellular components exposed during homogenization or it may release lectin bound to endogenous

membrane-bound receptors. This situation has also been found to be true for other soluble lectins (Mbamalu, 1985; Harrison et al, 1984).

The experiments presented here show that the Xenopus lectin is developmentally regulated during early embryonic stages. The level of activity is significantly higher at the gastrula stage than at the cleavage, blastula or neurula stages. This is an important finding because gastrulation is the time of active cell movements and these results suggest that this lectin may be involved somehow in the regulation of these movements. This is the first clear demonstration that high lectin activity correlates with the time of gastrulation in any species. There have been other reports of the developmental regulation of lectins during differentiation. One example is the slime mold lectin which reaches a peak of activity as individual cells aggregate to form a fruiting structure (Rosen et al, 1973). Another example is the erythroid developmental agglutinin (EDA) which is present in bone marrow on the surface of developing erythroblasts but not on mature reticulocytes (Catt et al, 1984).

Roberson and Barondes (1982) detected a similar lectin activity in Xenopus oocytes, blastula-stage embryos and in adult liver. In their study, lectin from oocytes and embryos had the same saccharide specificity as the

lectin reported in this work. However, the specific activity reported for their lectin extract is not as high as the one reported here, possibly because of differences used for extraction and purification. In particular, these authors used an acetone extraction of the crude material and they purified the lectin on a melibiose-linked affinity column. A galactose-specific lectin activity is also present in cortical granules of unfertilized Xenopus eggs (Wyrick et al, 1974) but since this activity has not been characterized, it is not possible to say if the cortical granule lectin is the same as the embryonic lectin. Other lectins reported in amphibian embryos include a lectin in the oocytes of Rana japonica which agglutinates Ehrlich ascites cells (Sakikibara et al, 1979); a lectin in oocytes of Rana catesbiana which agglutinates tumor cells and sialidase-treated human erythrocytes (Nitta et al, 1984); and a β-galactoside binding lectin from Rana catesbiana tadpoles which agglutinates A, B and O human erythrocytes (Nitta et al, 1983). A detergent-soluble lectin with oligomannosyl-binding properties has been described from early cleavage embryos of Rana pipiens (Roberson and Armstrong, 1980). The structural and functional relationships between these various lectins is not clear at the present time.

2. LECTIN SACCHARIDE SPECIFICITY

Since the embryonic Xenopus lectin agglutinates erythrocytes, it is in all probability at least divalent with respect to its carbohydrate binding sites. The specificity of this lectin was defined in terms of the saccharide which was able to inhibit agglutination at the lowest concentration. The best inhibitor in solution, however, is not necessarily identical to the carbohydrate which acts as a receptor on the cell surface. The interpretation of this data is complicated by the fact that lectins interact with complex oligosaccharides and this interaction may involve internal as well as terminal residues. The three-dimensional presentation of the terminal groups on the surface of cells is an important factor influencing binding of a lectin, as is the nature of side-chains, saccharide linkages and the steric effects of adjacent molecules. Thus, a rather weak soluble inhibitor may in fact be a strong lectin receptor in vivo under the right set of conditions. In spite of these reservations, saccharide inhibition studies provide some useful information since they make it possible to compare lectins from different tissues on the basis of their specificity for simple saccharides in solution. In this study, Xenopus lectin isolated from cleavage, blastula, gastrula and neurula embryos showed the same characteristics with

respect to saccharide specificity, suggesting that embryos at these stages contain the same lectin molecule. The embryonic lectin in all stages showed a specificity for terminal galactose groups. Thus, lactose and thiodigalactoside (TDG) are the most potent lectin inhibitors. Compared to other saccharides, they block lectin-mediated agglutination at the lowest relative concentrations. Both of these disaccharides have terminal non-reducing β-D-galactosyl residues. The fact that lactose and TDG are better inhibitors than galactose suggests that the active site of the lectin can accommodate more than one saccharide residue. Similar results have been found with endogenous lectins in unincubated and gastrulating chick blastoderms (Cook et al, 1979), differentiating tissues of later chick embryos (Den and Malinzak, 1977; Nowak et al, 1977; Kobiler and Barondes, 1977) and the galaptins in general. Unlike the above examples, however, the Xenopus lectin appears to lack strict anomeric specificity. It is inhibited to a greater extent by methylα-D-galactopyranoside than by methylβ-D-galactopyranoside. However, lactose and TDG are better inhibitors than melibiose, αDGal(1-6)DGlc. This latter result seems to indicate that the saccharide linkage is an important factor in lectin-carbohydrate binding. No significant inhibition was observed with N-acetylgalactosamine, methylα-D-manno-

pyranoside or fucose. This suggests that the hydroxyl groups at carbon position 2, carbon position 4 and carbon position 6 of galactose could be important to lectin binding.

The specificity of the Xenopus embryonic lectin was further examined by using immunoadsorbent materials possessing bound monosaccharide or disaccharide residues. The use of immunoadsorbents confirmed the specificity of this lectin for α- and β-galactose. Of the sugars tested, the disaccharide DGal(α1-3)DGal(β1-R) was the most effective inhibitor of the agglutinating activity. This particular disaccharide occurs naturally on the surface of human B erythrocytes (Lemieux, 1978). It has also been reported on the surface of rabbit erythrocytes (Honma et al, 1981). In this species, it forms the terminal non-reducing sequence of a large molecular weight, water soluble macroglycolipid. This molecule contains carbohydrate, sphingosine and fatty acids, but no amino acids. Possibly, this macroglycolipid may be the receptor for the Xenopus lectin in the haemagglutination assay. Honma et al (1981) also point out that a ceramide comprising a major proportion of the glycolipid fraction from rabbit erythrocyte membranes possesses the terminal trisaccharide Gal(α1-3)Gal(β1-4)GlcNAc. It is not known whether lipids of this nature exist in amphibian embryonic cells, either

on the cell surface as components of the plasma membrane or as part of the yolk material. The investigations with immunoadsorbents showed that DGal(α1-4)DGalβ1-R and DGal(β1-3)DGalNAcα1-R also effectively bound soluble lectin. The fact that DGal(β1-3)DGalNAcα1-R was a much better inhibitor than DGal(β1-3)DGlcNAcβ1-R suggests again that the lectin binding site may recognize at least two sugar residues.

The conclusions drawn from the saccharide inhibition tests are that crude lectin extracted from cleavage, blastula, gastrula and neurula stages of Xenopus embryos exhibit similar specificity for α- and β-galactosides. The best inhibitor of the embryonic lectin is the disaccharide DGal(α1-3)DGalβ1-R. This structure may resemble the endogenous lectin receptor in amphibian embryonic cells.

The Xenopus lectin differs from galaptins in that it requires the presence of calcium ions for its activity. The presence of EDTA or EGTA reversibly inhibits lectin mediated haemagglutination. A requirement for calcium has also been reported for other lectins, most notably the mammalian hepatic lectin (Stockert et al, 1977). Some plant lectins, including Con A also require bound calcium for carbohydrate binding activity. In the case of Con A, calcium stabilizes the subunits by reducing repulsion

between closely associated carboxyl groups (Goldstein and Hayes, 1978). Presumably, calcium also stabilizes the binding site of the Xenopus embryonic lectin, an observation which could have functional significance since early embryonic cells are easily dissociated in calcium-free medium. Brick and Weinberger (1984) have reported that an increase in the level of calcium ions is associated with the initiation of the dorsal blastopore lip. Calcium ions released from internal stores may be a normal regulatory mechanism in gastrulation. It is not known how fluctuating calcium levels might effect lectin activity in the embryo. As discussed by the above authors, calcium is involved in both the formation and function of submembrane microfilaments and in adhesive interactions between a cell and its substratum. Both of these processes are involved in the changes in shape and arrangement of cells during gastrular movements. This is also the stage when lectin activity is highest.

3. MOLECULAR PROPERTIES OF THE XENOPUS EMBRYONIC LECTIN

Affinity batch adsorption or affinity chromatography, followed by elution of the lectin activity with lactose, were the methods chosen for lectin purification. The average yield of lectin purified by affinity batch

adsorption was 7.2% of the total soluble extractable protein from blastula stage embryos and 8.8% of the total soluble protein from gastrula embryos. These figures are within the range reported for other soluble lectins. For example, the lectin from Dictyostelium is about 1% of the total soluble protein in aggregating cells (Barondes, 1983). Conrad et al (1984) have reported that the lectins of the sponge, Geodia cydonium comprise up to 14% of the soluble protein in this organism. According to Barondes (1983) this high value indicates that these lectins most likely have a structural role, since a protein with an enzymatic function would be expected to be present in minute quantities.

Purified lectin obtained by batch adsorption and examined by SDS-PAGE resulted in a number of bands, which varied between preparations from 2 to 9. A diffuse band between 37,000 and 45,000 molecular weight and a discrete band of 65,000 molecular weight were among the most frequent components. Purified lectin obtained by APL-Sepharose chromatography and examined by SDS-PAGE resulted in the appearance of both the discrete band of 65,000 molecular weight and the diffuse band of lower molecular weight. The diffuse band stained with periodic acid Schiff (PAS) stain, indicating that it contains bound carbohydrate. This band probably corresponds to the band

identified as the oocyte lectin by Roberson and Barondes (1982).

When purified lectin extracts were treated with chloroform-methanol, lectin activity remained in the aqueous phase. When aqueous phase material was lyophilized and examined by SDS-PAGE, a single band of molecular weight 65,500 was resolved. This band did not stain with PAS, and therefore is not likely a glycoprotein, although it is possible that the amount of carbohydrate is too small to be detected under the conditions used here. The conclusions from these experiments is that the endogenous galactose-binding lectin from Xenopus embryos is a protein of molecular weight 65,500 $\pm$ 2,780. This protein can be separated from closely associated molecules by chloroform-methanol treatment without reducing lectin activity. This strongly suggests that the 43,000 - 45,000 molecular weight material extracted from oocytes (Roberson and Baróndes, 1982) is a contaminant of the Xenopus lectin. Recently, Roberson et al (1985) reported the isolation of a lectin from the serum of adult female frogs which also had a galactose specificity and a molecular weight of 69,000. This lectin may be the same as the one reported here.

The other bands resolved by SDS-PAGE in samples not treated with chloroform-methanol may represent lipopro-

tein, glycoprotein or glycolipid material that is associated with the lectin either *in vivo* or after cell disruption by homogenization. High levels of lipoproteins are present in amphibian embryonic cells in the form of yolk, and this material might contribute to the pattern of bands. An association of the endogenous slime mold lectin with lipid material has previously been reported (Bartles et al, 1979). The diffuse appearance of many of these bands is further evidence that this material is not solely protein. When components of erythrocyte membranes are separated by PAGE and stained with Coomassie blue, the glycolipid and glycoprotein material appear as diffuse bands (Hamaguchi and Cleve, 1972). Dzandu et al (1984) report that staining lipid material with silver also results in diffuse bands. In comparison, non-glycosylated proteins are resolved as discrete bands under these conditions. The bands associated with the purified lectin protein which had not been treated with chloroform-methanol may represent some endogenous receptors of the lectin. They were described in the results section because of this possibility.

Two proteins with similar but distinct isoelectric points at 4.3 and 4.5 were resolved upon isoelectric focussing of purified *Xenopus* lectin. Roberson and Barondes (1982) reported the presence of at least four

diffuse bands of Xenopus oocyte lectin ranging in pH from 4.4 to 4.9. Under the conditions used here involving detergent-free aqueous solubilization of the samples, it is possible that contaminating material is not solubilized and does not enter the gel. In the experiments reported here, the two bands resolved by isoelectric focussing may represent isolectins. Similar isolectins have been reported for the endogenous lectin of rabbit bone marrow and in Dictyostelium discoideum, where isolectins of discoidin are the product of a four member gene family (Bartles et al, 1982). In Xenopus embryos, it is not known whether the isolectins are products of separate genes or are produced by post-translational modification of a single gene product. The four isolectins reported in oocytes may also reflect different gene expression in these cells compared to embryos.

Initial attempts to characterize this lectin in aqueous medium on a column of Sephacryl S-300 SF resulted in the activity being eluted as a continuum of low to high molecular weight species (25,000 to 250,000). This observation is perhaps due to the combined effects of self-association of the lectin molecules and to non-specific interactions with the column matrix. In aqueous media containing lactose, the lectin activity which had not been treated with chloroform-methanol eluted as an aggregate of

490,000 molecular weight. This value agrees with the molecular weight reported by Roberson and Barondes (1982) for oocyte lectin. The results reported here indicate that, in the absence of lactose, the lectin aggregates into various molecular sizes. When saccharide-binding sites are fully occupied, however, one aggregate size is formed at 490,000 molecular weight. Lectin extracts treated with chloroform-methanol and chromatographed under the same conditions eluted as an aggregate with a molecular weight of 371,000. Again this suggests that lipid or glycolipid material removed by chloroform-methanol treatment either associates with the lectin molecule under aqueous conditions or otherwise influences the aggregation of the lectin subunits.

The results of the analysis of the amino acid composition of the embryonic lectin are presented. The relative amounts of the amino acids do not appear to be unusual. No hexosamines were detected in this analysis supporting the conclusion that this protein is not a glycoprotein. The sample used for this test was resolved in a single band at 65,000 molecular weight on PAGE. Recently, Roberson et al (1985) reported the amino acid content of purified lectin from both adult female serum (molecular weight 69,000) and oocytes (molecular weight 43,000 and 45,000). The relative amino acid content

reported here for Xenopus embryonic lectin is more similar to the content reported by Roberson et al (1985) for the serum lectin than for the oocyte lectin. These results further support the conclusion that the 43,000 - 45,000 molecular weight material extracted from oocytes is a contaminant of the Xenopus lectin.

4. LECTIN LOCALIZATION

4.1 Characterization of the Antiserum:

Two purified lectin samples which had been treated with chloroform-methanol were injected into the rabbit. The first sample used for injection had resulted in a single band at 65,500 molecular weight when examined by SDS-PAGE. The second sample used for injection was obtained from a different batch of gastrula embryos. This lectin sample was purified by the same method as the first sample but was not examined by SDS-PAGE. The polyvalent antibodies obtained from the rabbit were characterized in several ways. Agar diffusion tests involving crude lectin extract or the purified lectin sample used for the second injection gave rise to a single precipitin line. When the same lectin sample or a crude lectin extract were separated by PAGE and used for immunoblotting, a single diffuse band between 37,000 and 42,000 molecular weight were

stained by the antiserum in both preparations. No antiserum staining was observed when protein blotting and immunoblotting was conducted on pure lectin samples showing a single band at 65,500 molecular weight on SDS-PAGE gels. This same pure sample, dissolved in TRIS-buffered saline and dotted on nitrocellulose, was stained by the polyvalent antiserum. Also, pure lectin protein dissolved and incubated in the sample buffer under the same conditions used for SDS-PAGE, does not stain with the immunodotting procedure. These results suggest that the antiserum interacts with the 37,000 to 45,000 lectin-associated protein as well as the 65,000 molecular weight lectin. The lectin may be denatured by the procedures used in the preparation of SDS-PAGE samples, and the epitopes no longer available for reaction with the antiserum. This phenomenon has been reported previously for monoclonal antibodies (Bers and Garfin, 1985). Dot immunoblotting indicated that the purified 65,500 molecular weight protein was recognized by the antiserum at a concentration as low as 1.25 ng/µl.

4.2 Immunohistochemistry:

Although control sections showed some staining, sections of early embryos stained with the antiserum

followed by preformed glucose oxidase-antiglucose oxidase complexes showed increased levels of staining. This staining was found extensively on the surface of large, outer blastomeres, in lengthening cleavage furrows and to a lesser extent between the membranes of deeper blastomeres. Pigment granules are also present in regions of increased staining, making the interpretation of these sections somewhat difficult.

Lectin localization was also identified in frozen or paraformaldehyde-fixed sections treated with antilectin antiserum and stained with fluorescent-labelled anti-rabbit antibodies. In cleavage and blastula stage embryos, the lectin was located in the vitelline envelope, in cleavage furrows, in extracellular material below the vitelline envelope on the surface of outer blastomeres and in some intracellular deposits associated with the external surface of the yolk platelets. Staining was also found in nascent blastocoele cavities and, in some cells, stained material appeared to be in the process of being either secreted or taken in by pinocytosis. Sections stained with pre-immune serum showed no bright fluorescent staining but a uniform pale autofluorescence was observed.

The results reported above are preliminary. The staining reflects the localization of both the 45,000

molecular weight lectin and the 37,000 to 45,000 molecular weight associated material. At the present time it is not known if the 37,000 to 45,000 molecular weight material is associated with the cell surface or if it is localized in yolk granules. No firm conclusions can be drawn from this data on the changes which may occur in the localization of the embryonic lectin during early development since all stages have not as yet been examined. Studies designed to investigate these changes are currently in progress. Attempts thus far have focussed on the best methods of fixation and staining of the amphibian embryos, as well as the preparation of the antiserum.

5. LECTIN RECEPTOR

Preliminary results are presented here on experiments designed to identify the endogenous receptor for the Xenopus embryonic lectin. Detergent-solubilized, membrane material was fractionated on a column containing Sephacryl S-300 SF. A single fraction inhibited the lectin haemagglutinating activity. When examined by SDS-PAGE, this fraction resulted in the appearance of two bands. The first band corresponded in molecular weight to the Xenopus embryonic lectin and this band did not stain with PAS. The second band stained with PAS, indicating that it is glycosylated, and had a molecular weight between 9,700 and

11,000. This material is possibly a low molecular weight glycoprotein or a glycolipid. It is also possible that this fraction represents an endogenous membrane-bound receptor for the Xenopus lectin. The localization of this material in early embryos and the nature of the carbohydrate groups attached to this molecule are unknown at the present time.

6. LECTIN FUNCTION

Three possible general functions have been proposed for lectin-carbohydrate interactions. Firstly, they may be involved in host-parasite or host-symbiont relationships. For example, proteins with lectin properties mediate the mannose-sensitive adherence of gram-negative bacteria, such as Escherichia coli to mammalian epithelial cells (Eshdat and Sharon, 1984). Also, a multivalent lectin has been implicated in the recognition and attachment of root hairs of the white clover, Trifolium repens, to glycosylated receptors on the symbiont Rhizobium trifoli (Dazzo et al, 1982). Such a function seems unlikely for the Xenopus embryonic lectin, however. Amphibian embryos during the early stages of their development are covered by three layers of dense jelly which form the outermost barrier for interactions with infectious organisms or parasites. It seems likely,

therefore, that a protein responsible for the inactivation or binding of such organisms would be located in these outer jelly layers (Wyrick et al, 1974). Such is not the case for the embryonic lectin, which appears to be concentrated in the vitelline envelope, the blastocoele, cleavage furrows and to a lesser extent in intercellular deposits.

The second function attributed to lectins is an involvement in intracellular transport of glycoproteins or in receptor-mediated endocytosis (Harford et al, 1984). An example of a lectin of this type is the mammalian N-acetyl-galactosamine and galactose-binding lectin on the surface of hepatocytes, endothelial cells and liver macrophages (Kolb-Bachofen et al, 1984). Lectins with this function appear to be membrane-bound proteins which can only be solubilized by detergent extraction. In addition, they are located either intracellularly or as components of the plasma membrane. Although this is not the case for the Xenopus embryonic lectin, cells during early stages of embryogenesis in amphibians are actively breaking down yolk components. From the immunohistochemical studies presented here, some lectin appears to be associated with yolk granules and therefore a possible role for the lectin in transport of the breakdown products cannot at the present time be eliminated.

The third proposed function for lectins is to play a role in tissue organization and differentiation. Thus,

lectins are frequently identified as components of the extracellular matrix in developing tissues. Lectins of this type appear to be easily soluble and are found in intracellular stores as well as secreted into the extracellular matrix (Barondes, 1984). Several lectins of this type have previously been mentioned, including the galaptins. This group includes lectins which have been implicated in myoblast fusion (Barondes and Haywood-Reid, 1981) and in the organization of the lung elastic fibers (Cerra et al, 1984). Recently, a soluble lectin has been implicated in the organization of the epithelial lining of the yolk sac in the chick embryo (Mbamalu, 1985). It seems likely that the galactose-binding lectin from Xenopus embryos belongs to this functional group, although it is not strictly speaking a galaptin, since it has a higher molecular weight and a requirement for calcium ions. It is, however, highly soluble and is located in both intracellular deposits and in the extracellular matrix. Since the activity of this lectin peaks during gastrulation, it is possible that it is involved in the cellular movements and tissue reorganization taking place during this stage.

7. DIRECTIONS FOR FURTHER STUDY

Many questions regarding the Xenopus embryonic lectin remain to be answered. Attention should be directed to

the localization of the 65,500 molecular weight protein in sectioned materials of embryos at different developmental stages. By adsorbing the antiserum against the 37-45,000 molecular weight contaminant, a more specific antiserum preparation will hopefully soon be available.

It is difficult to discuss the possible roles of this lectin in gastrulation without knowing more about the endogenous receptor molecules with which it interacts. An endogenous receptor for this lectin has been demonstrated in this work to be present in the membrane fraction from gastrula cells. The nature of endogenous receptors and their locations need to be further investigated. It would also be interesting to determine if the lectin and receptor co-localize during stages of early embryonic development. An immunohistochemical study at the electron microscope level would address the question of where the lectin is located at the cellular level.

Little is known about the nature of the cell surface carbohydrates of amphibian cells. Since embryos of Xenopus laevis are more abundant than embryos of many other vertebrate species, and since carbohydrates appear to be necessary for cellular recognition during early development, this would appear to be an ideal system to investigate the involvement and organization of glycoconjugates and their carbohydrate-binding proteins.

Finally, a role for this lectin in morphogenetic movements is implied by the experiments described here. However, more evidence about the effect of this lectin and its saccharide inhibitors on the movements and adhesive contacts between these cells is necessary. This evidence could be provided by cell culture experiments such as the ones carried out by Milos and Zalik (1981, 1982) to study the role of the β-galactoside lectin in chick embryos.

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